

Lysophosphatidic Acid (LPA) Enhances the Metastatic Potential of Human Colon Carcinoma DLD1 Cells through LPA1¹

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ABSTRACT

Lysophosphatidic acid (LPA) is a lipid mediator with diverse effects on various cells. Here, we investigated the effects of LPA on human colon carcinoma DLD1 cells. Northern blot analysis revealed that DLD1 highly expressed LPA1/Edg-2 but showed only low expression of LPA2/Edg-4 and no expression of LPA3/Edg-7 at the mRNA level. Western blot analysis revealed that DLD1 cells highly expressed LPA1 at the protein level. Using the Boyden chamber assay, LPA markedly increased DLD1 cell migration at concentrations as low as 10 nM, with maximum stimulation at 100 nM (3.6-fold increase). Checkerboard analysis indicated that LPA stimulated both the chemotactic and chemokinetic migration of DLD1 cells. LPA induced a dose-dependent increase in the proliferation of DLD1 cells (3.2-fold increase at 20 μ M). Furthermore, LPA stimulated DLD1 cell adhesion to collagen type I (2.0-fold increase at 10 μ M) and also stimulated the secretion of both vascular endothelial growth factor (1.4-fold increase at 20 μ M) and interleukin 8 (19-fold increase at 20 μ M) by ELISA. In contrast, as for matrix metalloproteinase, LPA had no significant effect on pro-matrix metalloproteinase-2 secretion and its activation, as measured by Western blot analysis. Thus, LPA, at concentrations that are present physiologically, enhanced DLD1 cell migration, proliferation, adhesion, and secretion of angiogenic factors, all of which are crucial for cancer metastasis. In comparison, other human colon carcinoma cells (HT29 and WiDR) exclusively expressed LPA2. LPA enhanced their proliferation and secretion of angiogenic factors, whereas LPA did not enhance migration or adhesion. Our results suggest that LPA acts as a potent stimulator of colon cancer progression, although the binding to LPA1 and LPA2 induces slightly different responses.

INTRODUCTION

LPA³ is a potent mediator with a broad range of cellular responses, including smooth muscle cell contraction, platelet aggregation, neurite retraction/cell rounding, regulation of cell proliferation, protection from apoptosis, modulation of chemotaxis, and transcellular migration (1). Some of these cellular responses implicate LPA as a mediator of tumor progression. In fact, LPA is present at high levels in the plasma and ascitic fluid from ovarian cancer patients (2, 3) and is known to be an “ovarian cancer activating factor” (4). Recently, it has been reported that LPA binds specifically and with high affinity to the G protein-coupled receptors, LPA1/Edg-2, LPA2/Edg-4, and LPA3/Edg-7. LPA receptors differ with respect to their tissue distribution. LPA1 is widely expressed, especially in the brain (5); LPA2 is most

highly expressed in the testis and leukocytes, whereas LPA3 is most highly expressed in the kidney and prostate (5, 6). In some types of cells such as ovarian and thyroid cells, malignant transformation seems to result in new appearance, or often predominance, of one or more LPA receptors (7). However, the functions of these LPA receptors in tumor biology have not been satisfactorily examined. In the present study, we investigated the effects of LPA on human colon carcinoma DLD1 cells, which express LPA1 exclusively, and demonstrated that LPA stimulated their migration, proliferation, adhesion, and the secretion of both VEGF and IL-8.

MATERIALS AND METHODS

Materials. 1-Oleoyl-LPA was purchased from Sigma (St. Louis, MO). DLD1 cells were treated with: (a) PTX (Calbiochem, La Jolla, CA); (b) Y27632 (supplied by Mitsubishi Pharma Co., Osaka, Japan); and (c) LY294002 (Calbiochem). cDNA probes of LPA1 and LPA2 were described previously (8). The cDNA probe of LPA3 was provided by Dr. Hiroyuki Arai (University of Tokyo, Tokyo, Japan; Ref. 6). Rabbit polyclonal antihuman LPA1 antibody was purchased from Exalpha Biologicals, Inc. (Boston, MA). Mouse monoclonal antihuman MMP-2 antibody was purchased from Daiichi Fine Chemical Co. (Toyama, Japan). Mouse monoclonal antihuman I κ B α antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture. The human colon cancer cell line DLD1 was obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% FCS (Sigma), 100 units/ml penicillin, and 100 μ g/ml streptomycin (Life Technologies, Inc., Grand Island, NY).

Preparation of Total RNA and Northern Blot Analysis. Total RNA was isolated from DLD1 cells by the acid guanidine isothiocyanate/phenol/chloroform extraction method as described by Chomczynski and Sacchi (9). RNA was separated by 1.0% agarose-formaldehyde gel electrophoresis, transferred onto a nylon membrane (GeneScreen; Perkin-Elmer Life Sciences, Inc., Boston, MA), and hybridized with cDNA probes labeled with [³²P]dCTP (Perkin-Elmer Life Sciences) using a DNA labeling kit (Nippon Gene, Inc., Toyama, Japan).

Western Blot Analysis of LPA1. Protein of the membrane fraction was extracted from DLD1 cells as described previously (10). The protein was electrophoresed in SDS-15% polyacrylamide gel for 45 min at 200 V. Then, the protein was transferred onto an Immobilon transfer membrane (Millipore, Bedford, MA) for sequential incubation with 5% reconstituted nonfat milk powder to block unspecific sites, dilutions of rabbit polyclonal anti-LPA1 antibody, and horseradish peroxidase-labeled donkey antirabbit IgG before development with a standard enhanced chemiluminescence kit (Amersham, Inc., Buckinghamshire, United Kingdom).

Migration Assay. Chemotactic migration of cells in response to a gradient of LPA was measured in a modified Boyden chamber. In brief, a polycarbonate filter with 8- μ m pores (Neuro Probe, Gaithersburg, MD), which was coated with collagen type IV (Nitta Gelatin, Inc., Osaka, Japan), was placed on a 96-blind-well chamber (Neuro Probe) containing 1 nM to 10 μ M LPA, and DLD1 cells (1×10^5 cells in 200 μ l/well) were loaded into the upper chamber. Ligand solutions and the cell suspension were prepared in DMEM containing 0.1% fatty acid-free BSA (Sigma). Some cells were pretreated with 100 ng/ml PTX for 24 h, 10 μ M Y27632 for 30 min, or 50 μ M LY294002 for 30 min. After incubation at 37°C in 5% CO₂ for 3 h, the filter was disassembled. The cells on the filter were fixed with methanol and stained with a Diff-Quick staining kit (International Reagents Co., Kobe, Japan). The upper side of the filter was then scraped free of cells. The number of cells that migrated to the

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³ The abbreviations used are: LPA, lysophosphatidic acid; VEGF, vascular endothelial growth factor; IL, interleukin; MMP, matrix metalloproteinase; PTX, pertussis toxin; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; ROCK, Rho-associated coiled-coil-forming protein serine/threonine kinase; NF- κ B, nuclear factor κ B.

lower side of the filter was determined by measuring absorbance at 595 nm using a 96-well microplate reader, model 3550 (Bio-Rad Laboratories, Hercules, CA). Checkerboard assays were carried out as described above, except that various dilutions of LPA in DMEM with 0.1% fatty acid-free BSA were placed in the upper and/or lower chambers of the Boyden chamber.

Proliferation Assay. DLD1 cells (5×10^3 cells in 100 μ l/well) were seeded in a 96-well plate in DMEM containing 0.1% fatty acid-free BSA with various concentrations of LPA, which was added every 24 h. After incubation at 37°C in 5% CO₂ for 120 h, the number of living cells was measured using a MTS assay (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, MTS solution was added to each well, and the cells were incubated for an additional 3 h. The number of living cells was determined by measuring absorbance at 490 nm.

Cell Adhesion Assay. Collagen type I-coated 96-well plates (Asahi Glass Co., Tokyo, Japan) were blocked with 2% fatty acid-free BSA overnight. The cells (2×10^4 cells in 100 μ l/well) were suspended in DMEM containing 0.1% fatty acid-free BSA with various concentrations of LPA, added to each well, and incubated for 25 min at 37°C. Some cells were pretreated with 100 ng/ml PTX for 24 h. Plates were washed three times with PBS using a 96-well microplate sera washer, model MW-96R (Biotec Co., Tokyo, Japan), and the number of remaining adherent cells was measured by MTS assay as described above.

ELISA of VEGF and IL-8. Samples were obtained as described previously (11). Briefly, after reaching confluence, DLD1 cells were incubated in serum-free medium for 24 h. LPA was added at various concentrations to the culture, and incubation was carried out for another 24 h. The protein levels of both VEGF and IL-8 in the conditioned medium were determined using a Quantikine Immunoassay kit (R&D Systems, Inc., Minneapolis, MN).

Western Blot Analysis of MMP-2 and I κ B α . Samples for analysis of MMP-2 were obtained as described previously (12). Briefly, DLD1 cells were starved in serum-free medium overnight, and then LPA was added to the culture. After incubation for another 24 h with various concentrations of LPA, conditioned medium was normalized based on the cell number, concentrated 40-fold, and then analyzed by Western blot analysis as described above, using mouse monoclonal antihuman MMP-2 antibody. HT1080 (a human fibrosarcoma cell line) cell culture medium was used as a positive control for pro-MMP-2 and activated MMP-2. Samples for analysis of I κ B α were obtained as described previously (13). After incubation of starved DLD1 cells with 20 μ M LPA for various times after the start of LPA stimulation, cellular protein lysates were obtained. Then, cell proteins (15 μ g/lane) were analyzed by Western blot analysis as described above, using mouse monoclonal antihuman I κ B α antibody.

RESULTS

Expression of LPA Receptors in DLD1 Cells. First, we examined the expression of LPA receptor mRNAs in DLD1 cells using Northern blot analysis. Among the three LPA receptors, LPA1 mRNA (transcript of about 4 kb) was highly expressed in DLD1 cells, whereas only low expression of LPA2 mRNA (transcripts of 1.8 and 2.8 kb) and no significant expression of LPA3 mRNA (transcript of about 4.3 kb) were detected, as shown in Fig. 1A. Then, we performed Western blot analysis on DLD1 cells using a polyclonal anti-LPA1 antibody, which showed high expression of LPA1 protein (Fig. 1B). Colon cancer Caco2 and COLO320 cells also expressed LPA1 mRNA significantly, whereas HT29, WiDR, and COLO201 cells did not (Fig. 1C). Thus, various colon cancer cells expressed variable levels of LPA1 without a consistent pattern. In contrast, most colon cancer cells, except COLO320 cells, expressed considerable levels of LPA2 and did not express LPA3 (Fig. 1C).

LPA Stimulates Both Chemotactic and Chemokinetic Motility of DLD1 Cells. DLD1 cells exhibited little spontaneous migration without LPA stimulation, and the addition of LPA to the lower chamber markedly induced DLD1 migration. The effect was significant at concentrations as low as 10 nM, with maximum stimulation at 100 nM (3.6 ± 0.2 -fold increase as compared with negative control). A further increase in the concentration of LPA produced less migra-

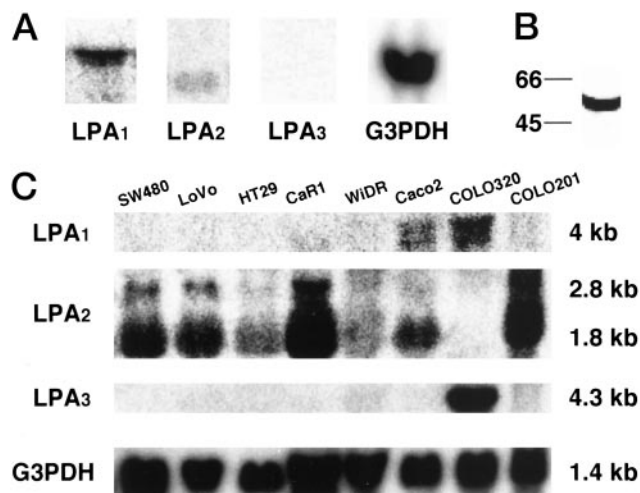


Fig. 1. A, Northern blot analysis of expression of LPA1, LPA2, and LPA3 mRNA by DLD1 cells. RNA extracted from cells was analyzed for mRNAs of LPA receptors. Glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) hybridization was used as a loading control. B, Western blot analysis of expression of LPA1 antigen protein by DLD1 cells. Protein extracted from cells was analyzed for LPA antigen protein. Rabbit monoclonal antihuman LPA1 antibody was used. The lines in the margin show the positions of the M_r 45,000 and M_r 66,000 protein molecular weight markers. C, Northern blot analysis of expression of LPA receptors by different colon cancer cells. RNA extracted from various colon cancer cells was analyzed for mRNAs of LPA receptors. Glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) hybridization was used as a loading control.

tion, producing a typical bell-shaped curve for chemotactic movement, as shown in Fig. 2A.

We next determined whether the effect of LPA was mediated by enhanced directed migration (chemotaxis) or by increased random motility (chemokinesis) by checkerboard assays. The greatest number of cells migrated along the chemotactic gradient of LPA placed in the lower chamber. LPA also enhanced migration in the absence of a gradient (equal concentrations in the upper and lower chambers), indicating a large chemokinetic component of the migration (Fig. 2B). These findings indicated that LPA stimulates both chemotactic and chemokinetic responses.

Inhibitors of Signaling Molecules Decreased LPA-induced Migration to Various Degrees. To understand the downstream signaling pathways stimulated by LPA that lead to DLD1 migration, we tested several agents that disturb signal transduction. To investigate the contribution of heterotrimeric G protein to LPA-induced DLD1 migration, we pretreated DLD1 cells with PTX (100 ng/ml, 24 h). As shown in Fig. 2C, PTX significantly inhibited LPA-induced DLD1 cell migration ($79 \pm 19\%$ inhibition). We next examined the functions of a major effector kinase of Rho, ROCK. Incubation with Y27632 (10 μ M, 30 min), an inhibitor of ROCK, produced a substantial decrease in LPA-induced migration ($45 \pm 10\%$ inhibition, Fig. 2C). In contrast, pretreatment with LY294002 (50 μ M, 30 min), an inhibitor of phosphatidylinositol 3'-kinase, did not cause a significant decrease in migration (Fig. 2C). Taken together, these findings strongly suggest the critical participation of G_i protein and Rho GTPase signaling pathways in LPA-enhanced migration of DLD1 cells.

LPA Stimulates Proliferation of DLD1 Cells. LPA has previously been shown to be a potent mitogen for some cell types, but not for others such as human umbilical vein endothelial cells and rat intestinal epithelial cells (IEC-6; Refs. 14 and 15). We examined the effects of LPA on proliferation of DLD1 cells. LPA induced a dose-dependent increase in proliferation as measured by MTS assay (Fig. 3). LPA showed significant growth stimulation at 20 nM and had stronger effects at even higher concentrations (3.2 ± 0.1 -fold increase at 20 μ M, compared with control), which was in clear contrast to the migration assay.

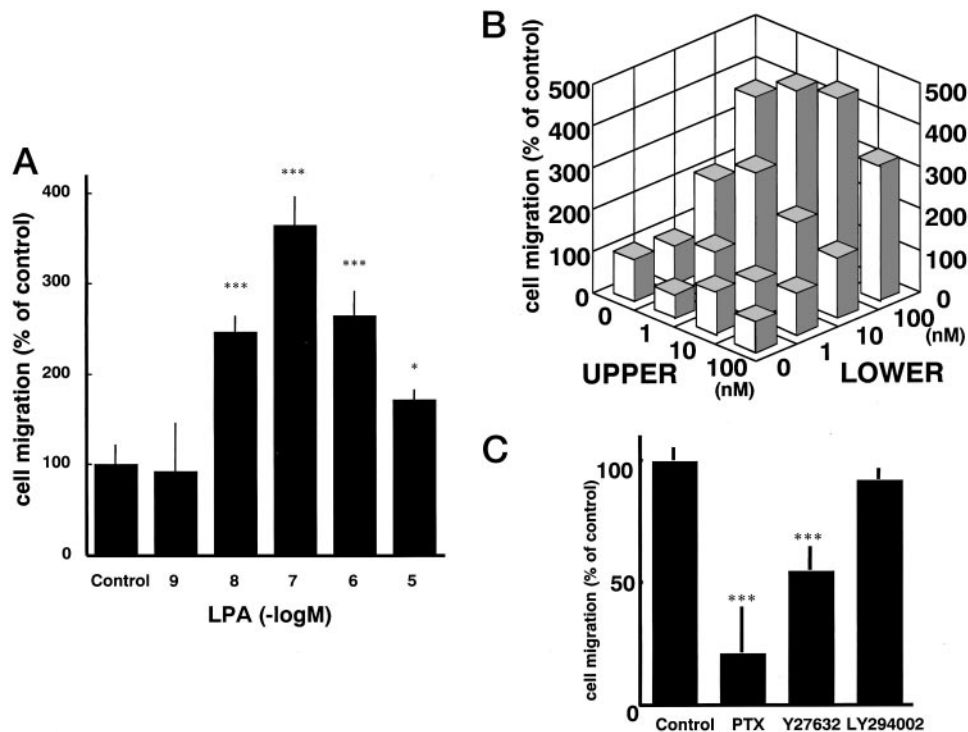


Fig. 2. *A*, Migration assay showing effect of LPA on DLD1 cells. DLD1 cells were allowed to migrate toward the indicated concentration of LPA, and chemotaxis was measured as described in "Materials and Methods." Data are expressed as a percentage of control, where control indicates cell migration in the absence of LPA (100%). Columns indicate the mean of three studies performed in triplicate; bars indicate SD. * and *** indicate significance at $P < 0.05$ and $P < 0.001$, respectively, compared with control by one-way ANOVA combined with Bonferroni's test. *B*, Checkerboard analyses of DLD1 migration induced by LPA. Cell migration assays were also performed as described in "Materials and Methods," with various concentrations of LPA added to the upper and lower chambers. Columns indicate the mean of triplicate determinations and are representative of three independent experiments with similar results. Data are expressed as a percentage of control, where control indicates cell migration in the absence of LPA in both the upper and lower chambers (100%). *C*, Effect of several inhibitors of signaling molecules on LPA-stimulated DLD1 cell migration. DLD1 cell migration to LPA (100 nM) was assayed after incubation with PTX, which inactivates G_i protein (100 ng/ml, 24 h), ROCK inhibitor Y27632 (10 μ M, 30 min), or phosphatidylinositol 3'-kinase inhibitor LY294002 (50 μ M, 30 min). Data are expressed as a percentage of control, where control indicates cell migration toward the lower chamber containing 100 nM LPA in the absence of inhibitors (100%). Columns indicate the mean of three studies performed in triplicate; bars indicate SD. *** indicates significance at $P < 0.001$ compared with control by one-way ANOVA combined with Bonferroni's test.

LPA Enhances DLD1 Cell Adhesion to Collagen Type I. Adhesion is the first step in the metastatic cascade; therefore, we addressed the role of LPA in cell adhesion. At the concentration that induced cell migration (10 nM to 1 μ M), LPA did not show any significant effect on cell adhesion. However, LPA at 10 μ M clearly enhanced DLD1 cell adhesion to collagen type I (2.0 \pm 0.5-fold increase as compared with control; Fig. 4A), and the effect was

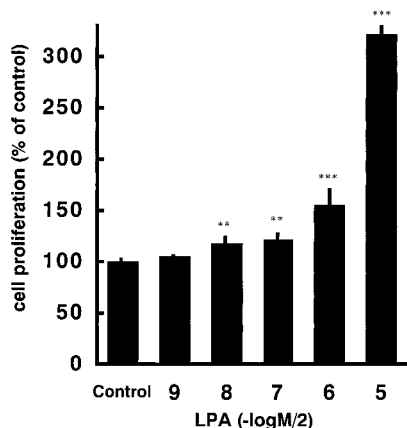


Fig. 3. Proliferation assay showing the effect of LPA on DLD1 cells. DLD1 cells were seeded with the indicated concentrations of LPA, and, after incubation for 120 h, cell proliferation was measured using a MTS assay. Data are expressed as a percentage of control, where control indicates cell proliferation in the absence of LPA (100%). Columns indicate the mean of three studies performed in triplicate; bars indicate SD. ** and *** indicate significance at $P < 0.01$, and $P < 0.001$, respectively, compared with control by one-way ANOVA combined with Bonferroni's test.

partially inhibited by PTX pretreatment (39 \pm 18% inhibition, Fig. 4A).

LPA Induced Secretion of Both VEGF and IL-8 Protein. Angiogenesis is necessary for tumor growth, and it depends on the production of angiogenic factors such as VEGF and IL-8. LPA has been reported to induce mRNA expression and protein secretion of both VEGF and IL-8 in human ovarian cancer cells (11, 16). We examined the effect of LPA on both VEGF and IL-8 secretion in DLD1 cells. LPA induced a significant increase in VEGF secretion at 20 μ M (1.4-fold increase as compared with control), whereas there were no significant increase or decrease at concentrations less than 2 μ M (Fig. 4B). In contrast, LPA induced a dose-dependent increase in IL-8 secretion, showing a significant effect at a concentration as low as 20 nM and stronger effects at higher concentrations (19-fold increase at 20 μ M, compared with control; Fig. 4C).

LPA Did Not Significantly Alter MMP-2 Secretion or Activation. Enzymatic degradation of the extracellular matrix is another important step in tumor cell invasion. We examined the effect of LPA on MMP-2 secretion and its activation in DLD1 cells, by Western blot analysis. However, expression of the pro-form of MMP-2 as well as the active form of MMP-2 was not changed by LPA over a wide range of LPA concentrations (2 nM to 20 μ M; Fig. 4D).

LPA Induced Degradation of I κ B α . IL-8 production is tightly regulated at several levels, particularly at the transcriptional level, to prevent aberrant production (17). To assess the mechanism of LPA-induced IL-8 secretion, we investigated whether LPA activates NF- κ B in DLD1 cells. By Western blot analysis, we examined LPA-induced degradation of I κ B α , an inhibitory protein whose proteolysis would

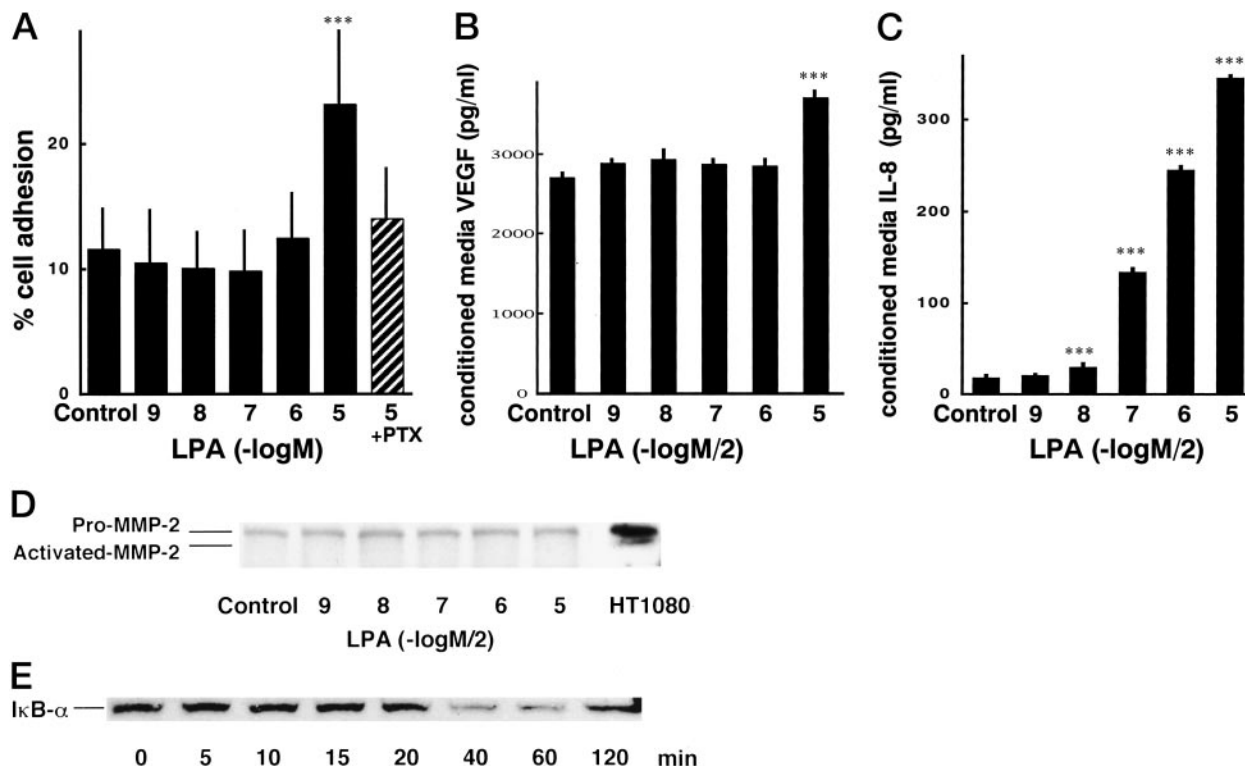


Fig. 4. *A*, Cell adhesion assay showing the effect of LPA on DLD1 cells. DLD1 cells were seeded on collagen type I-coated 96-well plates with the indicated concentrations of LPA, and after incubation for 25 min, plates were washed three times with PBS, and the number of remaining adherent cells was measured using a MTS assay. Some cells were pretreated with 100 ng/ml PTX for 24 h. Data are expressed as a percentage of adherent cell number compared with seeded cell number (100%). Each column indicates the mean of 6 wells; bars indicate SD. *** indicates significance at $P < 0.001$ compared with control by one-way ANOVA combined with Bonferroni's test. *B*, ELISA analysis of secreted VEGF protein concentration, showing the effect of LPA on DLD1 cells. VEGF protein level in the conditioned medium of DLD1 cells, in the presence of various concentrations of LPA, was determined by ELISA. Results are from one experiment with triplicate samples and are representative of three experiments; bars indicate SD. *** indicates significance at $P < 0.001$ compared with control by one-way ANOVA combined with Bonferroni's test. *C*, ELISA analysis of secreted IL-8 protein concentration, showing the effect of LPA on DLD1 cells. IL-8 protein level in the conditioned medium of DLD1 cells, in the presence of various concentrations of LPA, was determined by ELISA. Results are from one experiment with triplicate samples and are representative of three experiments; bars indicate SD. *** indicates significance at $P < 0.001$ compared with control by one-way ANOVA combined with Bonferroni's test. *D*, Western blot analysis of expression of pro-MMP-2 protein and activated MMP-2 protein showing the effect of LPA on DLD1 cells. Protein of concentrated condition medium was analyzed for pro-MMP-2 and activated MMP-2 protein. Mouse monoclonal antihuman MMP-2 antibody was used. HT1080 cell (a human fibrosarcoma cell line) culture medium was used as a positive control for pro-MMP-2 and activated MMP-2. *E*, Western blot analysis of expression of I κ B α protein, showing the effect of LPA on DLD1 cells. DLD1 cells were incubated with 20 μ M LPA for the indicated times. Cell extracts (15 μ g/lane) were prepared and analyzed for I κ B α . Mouse monoclonal anti-I κ B α antibody was used.

precede the translocation of NF- κ B to the nucleus. As shown in Fig. 4*E*, the level of I κ B α decreased slowly after stimulation, reaching a minimum at 40–60 min, and thereafter increased to near the control value.

Effects of LPA on Other Colon Cancer Cells through LPA2 Receptor. Most of the other different colon cancer cells expressed considerable levels of LPA2 mRNA as shown in Fig. 1*C*. Among them, HT29 cells and WiDR cells expressed LPA2 exclusively. Therefore, using these two colon cancer cells, we investigated the effects of LPA on colon cancer cells through the LPA2 receptor with the same assay that we performed in DLD1 cells. In HT29 and WiDR cells, LPA also enhanced their proliferation and secretion of angiogenic factors (Fig. 5, *A–C*). However, in contrast to DLD1 cells, neither cell migration nor adhesion in HT29 and WiDR cells was significantly enhanced by LPA under any experimental conditions (data not shown).

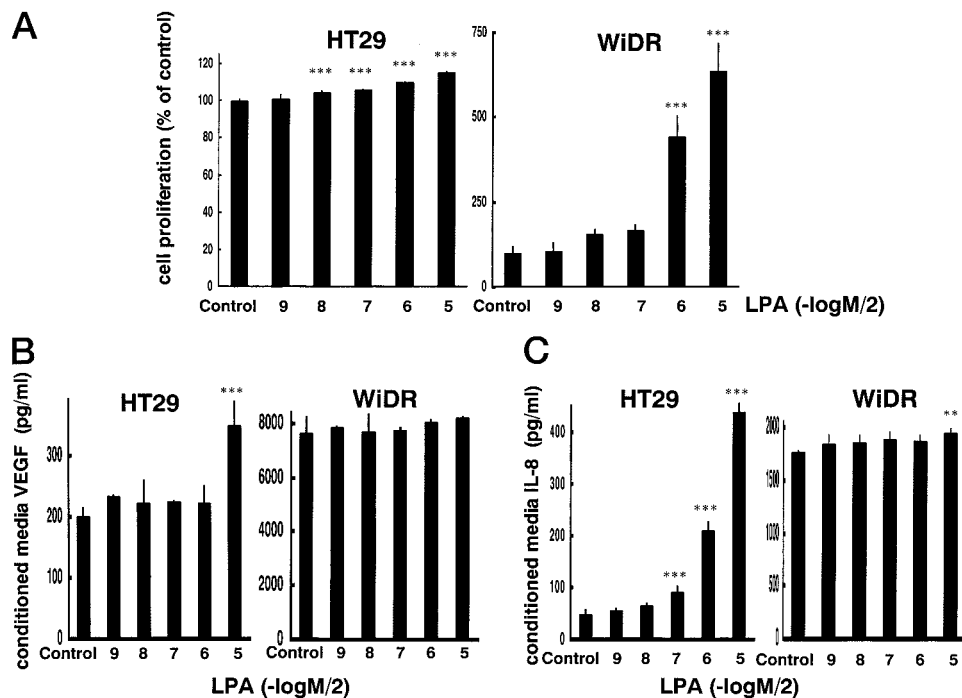
DISCUSSION

It has been shown that specific G protein-coupled receptors mediate the cellular effects of a natural phospholipid, LPA. At least three receptors, LPA1, LPA2, and LPA3, have been identified as cellular receptors for LPA (18). These consist of 364, 351, and 353 amino acids, respectively, and share 50–54% identical amino acids (18). The

tissue distribution differs markedly among the three LPA receptors (5). It has been suggested that malignant transformation results in new appearance or quantitative changes in the levels of LPA receptor expression, at least in some tumors. Whereas normal ovarian epithelial cells express LPA1 mRNA but have low levels of LPA2 and LPA3 mRNA, most ovarian cancer cells express elevated levels of LPA2 and LPA3 mRNA and variable levels of LPA1 mRNA without a consistent pattern (3, 10). In thyroid cells, LPA2 mRNA expression was increased 3-fold in differentiated thyroid cancer compared with normal thyroid or goiter (7). These data suggest that LPA2 (or LPA3) may be a key receptor involved in LPA stimulation of ovarian and thyroid tumor growth. On the other hand, it was reported that overexpression of LPA1 induced apoptosis and anoikis in ovarian cancer cells, implicating LPA1 as a negative growth receptor under some circumstances (19).

Cell migration is important not only in a variety of normal physiological processes, including embryogenesis, reproduction, inflammation, and wound healing, but also in pathological processes such as local tumor invasion and distant metastasis (14). LPA has been shown to be chemotactic for some endothelial cell types (20). On the other hand, chemotaxis of osteosarcoma cells toward platelet-derived growth factor is inhibited by the addition of LPA (21). Thus, LPA induces different migratory responses in different cell types. In our

Fig. 5. Effects of LPA on other colon cancer cells (HT29 and WiDR cells) through the LPA2 receptor. A, Proliferation assay showing the effect of LPA on HT29 and WiDR cells. The experimental procedure was as described in Fig. 3. Statistical analyses were performed as described in Fig. 3. *** indicates significance at $p < 0.001$. B, ELISA analysis of secreted VEGF protein concentration showing the effect of LPA on HT29 cells and WiDR cells. The experimental procedure was as described in Fig. 4B. Statistical analyses were performed as described in Fig. 4B. *** indicates significance at $p < 0.001$. C, ELISA analysis of secreted IL-8 protein concentration, showing the effect of LPA on HT29 and WiDR cells. The experimental procedure was as described in Fig. 4C. Statistical analyses were performed as described in Fig. 4C. ** and *** indicate significance at $p < 0.01$, and $p < 0.001$, respectively.



study, we found that LPA was a strong chemoattractant for DLD1. The Boyden chamber assay clearly revealed that LPA stimulated both chemotaxis and chemokinesis. In contrast to DLD1, HT29 and WiDR, which exclusively expressed LPA2, did not show chemotaxis to LPA. This suggests that LPA-induced migration is mainly dependent on a LPA1-mediated response. Our suggestion was supported by the finding that one LPA1-mediated response is Rho activation (22) and that Rho controls cell motility through reorganization of the actin cytoskeleton and regulation of actomyosin contractility (23).

Angiogenesis is also important in cancer progression. Here, we demonstrated that LPA strongly induced secretion of angiogenic factors in colon cancer cells. Our finding of LPA-induced degradation of $\text{I}\kappa\text{B}\alpha$ in DLD1 may partially explain the mechanism of LPA-induced IL-8 secretion. Degradation of $\text{I}\kappa\text{B}\alpha$ would proceed with NF- κB nuclear localization and transactivation of its target genes (24), and IL-8 is one of the many target genes of NF- κB (17, 25). Previous reports revealed that in a fibroblast cell line (Swiss 3T3) and in primary endothelial cells, LPA promotes activation of NF- κB (13, 26), although what LPA receptor mediated this response was not discussed. Sphingosine 1-phosphate, a structurally related lysophospholipid, has been shown to activate NF- κB through S1P3/Edg-3 and S1P2/Edg-5 but not through S1P1/Edg-1, suggesting an essential role of G_q to NF- κB activation through the Edg family (27). Because all three LPA receptors, LPA1, LPA2, and LPA3, were coupled to G_q (18), these data are compatible with our findings that LPA induced NF- κB activation and subsequent secretion of IL-8 in colon cancer cells through both LPA1 and LPA2. LPA also showed a significant effect on VEGF secretion, although the effect was not as impressive as that on IL-8. In the resting state, DLD1 secreted a considerable amount of VEGF but only a small amount of IL-8. This suggests the possibility that the stimulatory effect of LPA on VEGF secretion can be partially masked as compared with that on IL-8 secretion. Another possible explanation for this discrepancy could be that these phenomena are mediated by different signal transduction pathways. A previous report showed that transcription of VEGF was partially, but not totally, dependent on NF- κB in endothelial cells (28) and suggested that the VEGF promoter region contained no typical NF- κB binding

motif but might contain a NF- κB -like binding domain that caused a partial contribution to the effect of NF- κB on VEGF transcription (28). The same mechanism is suggested to work in our experimental system, resulting in the different responses between VEGF and IL-8 secretion.

Our study revealed that LPA enhanced the metastatic potential of colon cancer cells as well as ovarian cancer cells, which have been much reported (3, 10–12, 16). In this study, we focused mainly on DLD1, which expressed LPA1 exclusively. However, as shown in Fig. 1C, the expression level of LPA1 varied without a consistent pattern among various colon cancer cells, whereas a considerable level of LPA2 was expressed in most colon cancer cells. Therefore, DLD1 may be a unique type of human malignancy that expresses LPA1 exclusively, through which the metastatic potential is strongly enhanced. Our data on WiDR and HT29 indicated that LPA, when bound to LPA2, could induce proliferation and angiogenic factor secretion, whereas it did not stimulate adhesion and migration. Thus, LPA1 and LPA2 had different functional properties in colon cancer cells, as shown in Fig. 6. Our results suggest the possibility that the expression pattern of LPA receptors in colon cancer tissues might be related to their clinical characteristics through the different responses to LPA.

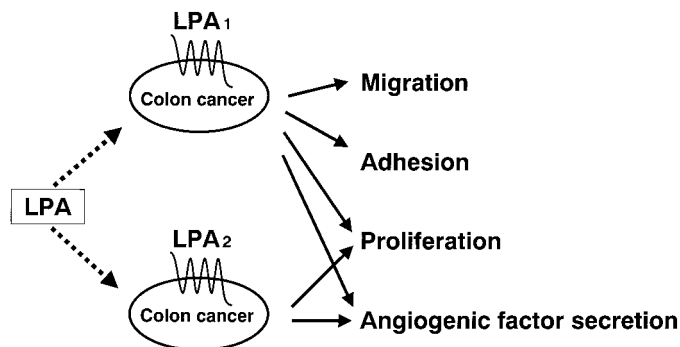


Fig. 6. Summary of the effects of LPA on colon cancer cells through LPA1 and LPA2.

A curious aspect of the reported LPA signaling is the wide variation in potency. Calcium mobilization, adenylate cyclase inhibition, and cytoskeletal rearrangements require nanomolar concentrations of LPA, whereas mitogenic and platelet aggregation responses require micromolar concentrations of LPA (29). As for effects on ovarian cancer cells, LPA markedly induced cell proliferation, cell adhesion, and secretion of both VEGF and IL-8 at micromolar concentrations (10–12, 16). These data are compatible with our findings that cell migration was most markedly stimulated at 10 nM, whereas cell proliferation, cell adhesion, and secretion of both VEGF and IL-8 were most markedly stimulated at 10–20 μ M in DLD1. Recently, Hooks *et al.* (29) proposed a new model for LPA signaling that includes an Edg-receptor-independent pathway that mediates mitogenesis and platelet aggregation. According to the required LPA concentrations, there may be a possibility that LPA-induced cell adhesion and secretion of both VEGF and IL-8 are also mediated by an Edg-receptor-independent pathway. However, this conundrum remains unsolved, and further investigation is needed.

Although the downstream biochemical events linking LPA to its pleomorphic activities are complex, and there are likely to be multiple levels of “cross-talk” among the signaling cascades activated by LPA, there could be a good opportunity to develop new effective therapy for cancer progression if we can modulate the activities of specific LPA receptors and downstream signal transduction pathways (3). Our findings revealed that inhibition of G_i and ROCK significantly decreased DLD1 migration, which is compatible with previous reports on the same inhibitors against lysophospholipid-induced endothelial cell chemotaxis (20, 30). Our findings also revealed that inhibition of G_i significantly decreased DLD1 adhesion. It would be ideal if new anticancer drugs could be identified and developed based on their ability to act as antagonists of growth-promoting LPA receptors or their downstream signaling molecules.

In summary, this study demonstrated that LPA stimulates cell migration, proliferation, adhesion, and VEGF and IL-8 secretion of DLD1, which suggests that LPA plays a significant role in malignant progression, including local invasion and distant metastasis, through the LPA1 receptor in certain colon cancer cells. Elucidation of the molecular mechanisms by which LPA stimulates cell migration, proliferation, adhesion, and secretion of angiogenic factors through LPA receptors might provide a clue for the development of new therapeutic agents.

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