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Cell Death and Survival

Molecular Cancer Research

Bioactive Lipids, LPC and LPA, Are Novel Prometastatic Factors and Their Tissue Levels Increase in Response to Radio/Chemotherapy

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Abstract

Bioactive lipids are fundamental mediators of a number of critical biologic processes such as inflammation, proliferation, and apoptosis. Rhabdomyosarcoma (RMS) is common in adolescence with histologic subtypes that favor metastasis. However, the factors that influence metastasis are not well appreciated. Here, it is shown that lysophosphatidylcholine (LPC) and its derivative, lysophosphatidic acid (LPA), strongly enhance motility and adhesion of human RMS cells. Importantly, these metastatic-associated phenotypes were observed at physiologic concentrations of these lipids, which naturally occur in biologic fluids. Moreover, the effects of these bioactive lipids were much stronger as compared with known peptide-based prometastatic factors in RMS, such as stromal-derived factor-1 or hepatocyte growth factor/scatter factor. Finally, both LPC and LPA levels were increased in several organs after γ-irradiation or chemotherapy, supporting the hypothesis that radio/chemotherapy induces an unwanted prometastatic environment in these organs.

Implications: LPC and LPA play a previously underappreciated role in dissemination of RMS and suggest that antimetastatic treatment with specific molecules blocking LPC/LPA activity should be part of standard radio/chemotherapy arsenal. *Mol Cancer Res; 12(11); 1560–73. ©2014 AACR.*

Introduction

Rhabdomyosarcoma (RMS) is the most common soft-tissue sarcoma of adolescence and childhood. There are two major histologic subtypes of this tumor, highly metastatic alveolar rhabdomyosarcoma (ARMS) and less-metastatic embryonal rhabdomyosarcoma (ERMS). RMS accounts for 5% of all malignant tumors in patients under 15 years of age (1, 2) and belongs to the family of so-called "small round blue tumor cells," which often infiltrate bone marrow and can resemble hematologic blasts. Thus, RMS cells may sometimes be misdiagnosed as acute leukemia cells (2).

At the molecular level, ARMS is characterized by the t(2;13)(q35;q14) translocation in 70% of cases or the variant t(1;13)(p36;q14) in a smaller percentage of cases (3). These translocations disrupt the *PAX3* and *PAX7* genes on chromosome 2 or 1, respectively, and the *FKHR* gene on chromosome 13, generating *PAX3–FKHR* and *PAX7–FKHR* fusion genes. These fusion genes encode the fusion proteins PAX3–FKHR and PAX7–FKHR, which have enhanced transcriptional activity compared with wild-type PAX3 and PAX7 and are postulated to play a role in cell survival and dysregulation of the cell cycle in ARMS (1). Recently, we also found that imprinting of the different methylated region at the *DLK1-GTL2* locus varies in association with the histologic subtype of RMS: embryonal rhabdomyosarcoma shows loss of imprinting, whereas alveolar tumors have erasure of imprinting at this locus (4). This difference provides evidence about different cellular origin of these tumors.

Several groups, including ourselves, identified several chemoattractants that lead to metastasis of RMS cells to bone marrow, including the α-chemokine stromal-derived factor 1 (SDF-1), hepatocyte grow factor/scatter factor (HGF/SF), and insulin-like growth factor type I and II (IGFI, IGF2), which are secreted by cells in the bone marrow microenvironment and play an important role in infiltration of bone marrow by RMS cells (5–8). Moreover, a robust chemotactic response to these factors is also observed in *in vitro* migration assays in which both SDF-1 and HGF/SF are used as chemoattractants at supraphysiologic concentrations (5, 6).

However, because the concentrations of these factors in biologic fluids and tissues are usually very low (9, 10), we began a search for other chemoattractants that could induce

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metastasis of RMS cells and identified two bioactive lipids, sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P), as factors involved in regulating metastatic behavior of RMS cells at physiologic concentrations (11). Moreover, we observed that both S1P and C1P are upregulated in bone marrow tissues after radio/chemotherapy, which supports the concept that one of the unwanted effects of radio/chemotherapy is induction of a prometastatic microenvironment in normal tissues damaged by treatment (11) and that factors induced by such treatment may be involved in metastasis of cancer cells resistant to the treatment (11, 12). On the basis of this concept, we became interested in two other bioactive lipids, namely, lysophosphatidylcholine (LPC) and its derivative generated by enzymatic action of autotaxin (ATX), lysophosphatidic acid (LPA; refs. 13, 14). As reported, LPA mediates metastases of several types of tumors via interactions with high-affinity G protein–coupled receptors (GPCR; ref. 15).

In this article, we present for the first time evidence that both LPC and LPA enhance motility and adhesive properties of RMS cells, and the levels of both bioactive lipids increase in several organs, including in bone marrow after γ-irradiation and vincristine treatment. Thus, we have identified LPC and LPA as novel prometastatic factors in human RMS cell lines and demonstrate that, like S1P and C1P, their tissue levels increase in response to radiotherapy. These observations not only shed more light on the role of bioactive lipids in the metastasis of cancer cells but should also prompt the development of new antimetastatic strategies to supplement treatment by radio/chemotherapy by targeting the metabolism and signaling actions of these bioactive lipids.

Material and Methods

Cell lines

We used several human RMS cell lines (gifts from Dr. Peter Houghton, World Children's Cancer Center, Columbus, OH and Prof. Fred Barr, University of Pennsylvania, Philadelphia, PA), including both ARMS (RH18, RH28, RH30, and RH41) and ERMS (JR, SMS-CTR, RD, and RH36) cell lines. All cell lines used in these studies were authenticated by short tandem repeat (STR) analysis. Obtained STR profile was compared either with STR profile of original cell lines obtained in Dr. Peter Houghton Laboratory or with published STR profile of cell lines. SMS-CTR and RH36 cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin, and 10 μg/mL streptomycin. All other cell lines were maintained in RPMI medium 1640, containing 10% FBS, 100 U/mL penicillin, and 10 μg/mL streptomycin. Stromal cells were maintained in DMEM containing 20% FBS, 100 U/mL penicillin, and 10 μg/mL streptomycin. All cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C, and the media were changed every 48 hours.

Murine bone marrow stromal cells

Bone marrow–derived stromal cells were expanded *ex vivo* from murine bone marrow mononuclear cells (BMMNC) as described (16). Briefly, BMMNCs were expanded in DMEM supplemented with 20% FBS and 50 U/mL penicillin/streptomycin for 7 to 10 days at 37°C in a 5%-CO_2 incubator.

Real-time quantitative reverse-transcription PCR

Total RNA was isolated from RMS cells with the RNeasy Kit (Qiagen). Human muscle RNA was obtained from Ambion. The RNA was reverse transcribed with MultiScribe reverse transcriptase, oligo(dT), and random-hexamer primer mix (Applied Biosystems). PCR was performed at 2 cycles of 2 minutes at 95°C, 1 minute at 60°C, and 1 minute at 72°C; 36 cycles of 30 seconds at 95°C, 1 minute at 60°C, and 1 minute at 72°C; and 1 cycle of 10 minutes at 72°C. Quantitative assessment of mRNA levels was done by real-time RT-PCR on an ABI 7500 instrument with Power SYBR Green PCR Master Mix reagent. Real-time conditions were as follows: 95°C (15 seconds), 40 cycles at 95°C (15 seconds), and 60°C (1 minute). According to melting point analysis, only one PCR product was amplified under these conditions. The relative quantity of a target, normalized to the endogenous β2-microglobulin gene as control and relative to a calibrator, is expressed as $2^{-\Delta\Delta Ct}$ (fold difference), where Ct is the threshold cycle, $\Delta Ct =$ (Ct of target genes) − (Ct of the endogenous control gene, β2-microglobulin), and $\Delta\Delta Ct =$ (ΔCt of samples for target gene) − (ΔCt of calibrator for the target gene). All primers that were used for RT-PCR or qRT-PCR are listed in Supplementary Table S1.

Cell proliferation

Cells were plated in culture flasks at an initial density of 1.25×10^4 cells/cm^2. After 24 hours, the medium was changed to new medium supplemented with 0.5% BSA, and cells were cultured in the presence or absence of LPA (0.1 or 1 μmol/L) or LPC (200, 20, or 2 μmol/L) with or without vincristine (0.5 or 5 μmol/L). Full medium (with 10% FBS) was treated as a positive control. The cell number was calculated at 24, 48, and 72 hours after the change of medium. At the indicated time points, cells were harvested from the culture plates by trypsinization and counted using Trypan Blue and a Neubauer chamber.

Apoptosis analysis

Cells were cultured under the same conditions as in the proliferation assay. After 72 hours from the start of LPA and vincristine administration, cells were trypsinized, centrifuged, and stained with Annexin V antibody and propidium iodide according to the manufacturer's protocol (Annexin V Apoptosis Detection kit; BD Biosciences). Analysis of stained cells was performed with a BD LSR II flow cytometer (BD Biosciences).

Chemotaxis assay

Chemotaxis assays were performed in a modified Boyden's chamber with 8-μm pore polycarbonate membrane inserts (Costar Transwell; Corning Costar) as described previously (17). In brief, cells detached with 0.05% trypsin were seeded

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into the upper chamber of an insert at a density of 3.5×10^4 in 110 μL. The lower chamber was filled with prewarmed culture medium containing test reagents. Medium supplemented with 0.5% BSA was used as a negative control. In some experiments, cells were pretreated with inhibitors U0126 (1 μmol/L; Promega), MK2206 (1 μmol/L; Selleckchem), or Ki16425 (10 μmol/L, Cayman Chemicals) for 15 minutes at 37°C. Inhibitors were also added to the lower chambers and were present throughout the experiment. The ATX inhibitor S32826 was obtained from Sigma-Aldrich. After 24 hours, the inserts were removed from the Transwell supports. The cells that had not migrated were scraped off with cotton wool from the upper membrane, and the cells that had transmigrated to the lower side of the membrane were fixed and stained with HEMA 3 (protocol, Fisher Scientific) and counted on the lower side of the membrane using an inverted microscope.

Phosphorylation of intracellular pathway proteins

RMS cell lines were kept overnight in RPMI medium containing low levels of BSA (0.5%) to render the cells quiescent. After the cells were stimulated with LPA (0.1 μmol/L) or LPC (20 μmol/L) at 37°C for 5 minutes or 2 hours, respectively, cells were lysed for 20 minutes on ice in RIPA lysis buffer containing protease and phosphatase inhibitors (Santa Cruz Biotechnology). The extracted proteins were separated on a 12% SDS-PAGE gel and transferred to a PVDF membrane. The phosphorylation of the serine/threonine kinase AKT (phospho-AKT473) and p44/42 mitogen-activated kinase (phospho-p44/42 MAPK) was detected by phospho-specific p44/42 MAPK mouse and rabbit polyclonal antibodies (Cell Signaling) with horseradish peroxidase–conjugated goat anti-mouse and anti-rabbit secondary antibodies (Santa Cruz Biotechnology). Equal loading in the lanes was evaluated by stripping the blots and reprobing with anti-p42/44 MAPK monoclonal antibody (clone no. 9102; Cell Signaling) and anti-AKT polyclonal antibody (Cell Signaling). The membranes were developed with an enhanced chemiluminescence reagent (Amersham Life Sciences), dried, and subsequently exposed to film (Hyperfilm; Amersham Life Sciences).

Rho activation assay

Rho activation assay was performed using the Rho Activation Assay Kit according to the manufacturer instruction (Millipore). Cells were made quiescent, and activation of Rho by LPA and LPC treatment has been performed as described above in the "Phosphorylation of intracellular pathway proteins" section.

Adhesion assay to fibronectin

Cells were made quiescent for 3 hours with 0.5% BSA in RPMI 1640 before incubation with LPA (0.1 μmol/L) or LPC (20 μmol/L) for 10 minutes. Subsequently, cell suspensions (5×10^3/100 μL) were added directly to 96-well plates covered with fibronectin and incubated for 5 minutes at 37°C. The wells were coated with fibronectin (10 μg/mL) overnight at 4°C and blocked with 0.5% BSA for 2 hours before the experiment. Following incubation, the plates were vigorously washed three times to remove nonadherent cells, and the number of adherent cells was counted using an inverted microscope.

Adhesion assay for bone marrow–derived stromal cells

RMS cells were labeled before assay with the fluorescent dye calcein AM and made quiescent by incubation for 3 hours at 37°C in RPMI 1640 medium supplemented with 0.5% BSA. The cells were then stimulated with LPA (0.1 μmol/L) or LPC (20 μmol/L) for 10 minutes at 37°C, then added to plates covered by mouse stromal cells, and incubated for 15 minutes at 37°C. After the nonadherent cells had been discarded, cells that adhered to the stromal cells were counted under a fluorescence microscope as described (5).

Preparation of conditioned media

Pathogen-free C57BL/6 mice were purchased from the NCI (Frederick, MD, USA), allowed to adapt for at least 2 weeks, and used for experiments at age of 7 to 8 weeks. Animal studies were approved by the Animal Care and Use Committee of the University of Louisville (Louisville, KY, USA). Mice (four per group) were irradiated with 250, 500, 1,000, or 1,500 cGy. Twenty-four hours later, bone marrow, liver, lungs, and plasma were isolated. Conditioned medium was obtained by 1-hour incubation of bone marrow, liver, or lung cells (mechanically homogenized 30 times using a syringe) in RPMI at 37°C. After centrifuging, the supernatant was used for further experiments. In studies with the chemotherapeutic agent vincristine, mice were injected intraperitoneally with 0.9% NaCl with (0.5 mg/kg or 2 mg/kg) or without vincristine. Twenty-four hours later, organs were isolated, and conditioned medium from various organs was prepared as described above.

Transplant of RMS cells into immunodeficient mice

To study the effects of the pharmacologic inhibition of LPA signaling on the metastasis of RMS *in vivo*, RH30 cells were either pretreated with Ki16245 (10 μmol/L) or vehicle alone for 1 hour. The cells were then washed and injected intravenously (3×10^6 per mouse) into SCID–Beige inbred mice (five mice per group) that were either untreated (control) or irradiated with 750 cGy 24 hours earlier. Marrows, livers, and lungs were removed 48 hours after injection of these cells, and the presence of RMS cells (i.e., murine–human chimerism) was evaluated as the difference in the level of human α-satellite DNA expression. DNA was amplified in the extracts isolated from bone marrow–, liver-, and lung-derived cells using real-time PCR. Briefly, DNA was isolated using the QIAamp DNA Mini Kit (Qiagen). Detection of human satellite and murine β-actin DNA levels was conducted using real-time PCR and an ABI Prism 7500 Sequence Detection System. A 25-μL reaction mixture containing 12.5 μL SYBR Green PCR Master Mix, 300 ng DNA template, 5′-ACC ACT CTG TGT CCT TCG TTC G-3′ forward and 5′-ACT GCG CTC TCA AAA GGA GTG T-3′ reverse primers for α-satellite DNA, and 5′-TTC AAT TCC AAC ACT GTC CTG TCT-3′ forward and 5′-CTG TGG AGT GAC TAA ATG GAA ACC-3′

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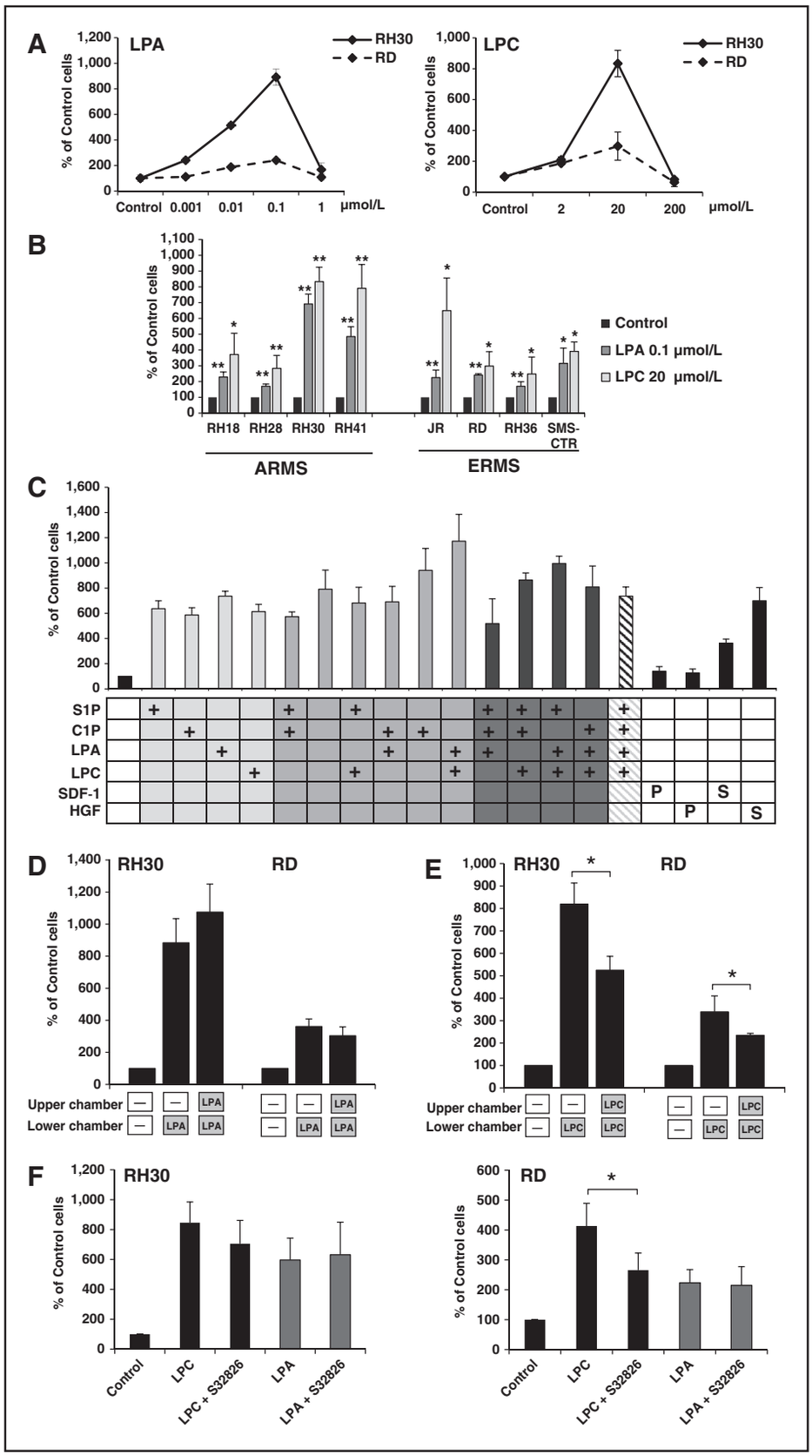

Figure 1. Bioactive lipids LPA and LPC are chemoattractants for RMS cells. A, concentration-dependent effect of LPA (left) and LPC (right) on migration of RH30 and RD cell lines. B, chemotaxis of different RMS cell lines across Transwell membranes in response to LPA (0.1 μmol/L) or LPC (20 μmol/L). C, chemotaxis of RH30 cells in response to S1P (1 μmol/L, considered to be a physiologic concentration), C1P (0.5 μmol/L, considered to be a physiologic concentration), LPA (0.1 μmol/L, considered to be a physiologic concentration), LPC (20 μmol/L), SDF1, and HGF in physiologic concentrations (5 ng/mL and 0.3 ng/mL, respectively; marked as P) or supraphysiologic concentrations (300 ng/mL or 10 ng/mL, respectively, marked as S). D and E, chemotaxis and chemokinesis of RH30 and RD cells in response to LPA (0.1 μmol/L) and LPC (20 μmol/L). F, chemotaxis of RH30 and RD cells to LPC in the presence of the ATX inhibitor S32826 (1 μmol/L). The chemotaxis assays were done at least twice in duplicate, with similar results. Results, mean ± SD, with a statistical significance relative to the control of *, $P < 0.05$ and **, $P < 0.01$.

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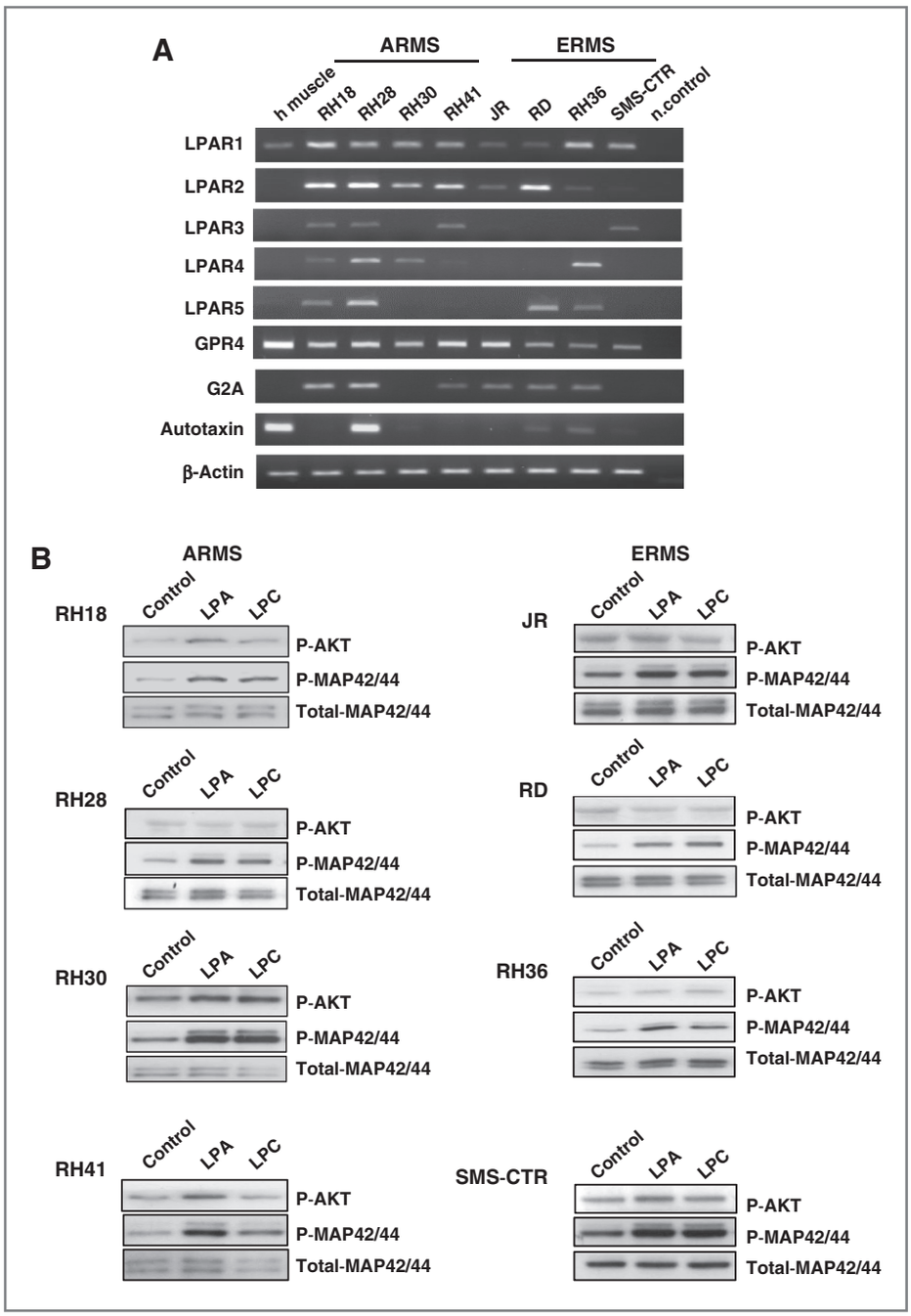

Figure 2. LPA and LPC activate MAPK intracellular pathway proteins and induce migration of human RMS cell lines through GPCRs. A, RT-PCR for LPA and LPC receptors revealed that RMS cells express these receptors. The experiment was repeated twice on two different batches of cells, with similar results. B, phosphorylation of p42/44 MAPK and AKT in human RMS cell lines stimulated for 5 minutes by LPA (0.1 μmol/L) or for 2 hours with LPC (20 μmol/L). Because both controls (5-minute stimulation and 2-hour stimulation in the presence of vehicle only) were similar, only one is shown. The experiment was repeated twice, with similar results, and representative Western blots are shown. (*Continued on the following page*.)

reverse primers for β-actin DNA was used. The Ct value was determined as before. The number of human cells present in the murine organs (the degree of chimerism) was calculated from the standard curve obtained by mixing different numbers of human cells with a constant number of murine cells.

Quantitation of LPA and LPC by tandem mass spectrometry

Lipids were quantitated by methods reported previously using high-performance liquid chromatography (HPLC) electrospray-selected ion-monitoring mode MS/MS assays performed on an AB Sciex 4000 Q-Trap instrument. In brief, instrument settings for each analyte were optimized by direct infusion and tuning. HPLC methods were identical to or adapted from our prior reports (18, 19). The instrument was operated in selected ion-monitoring mode to measure lipid-specific precursors and product ion pairs for C17-LPA (423.2/152.9) and C19-LPC (538.4/184.0). In each case, structurally related lipids were included during the sample extraction to monitor

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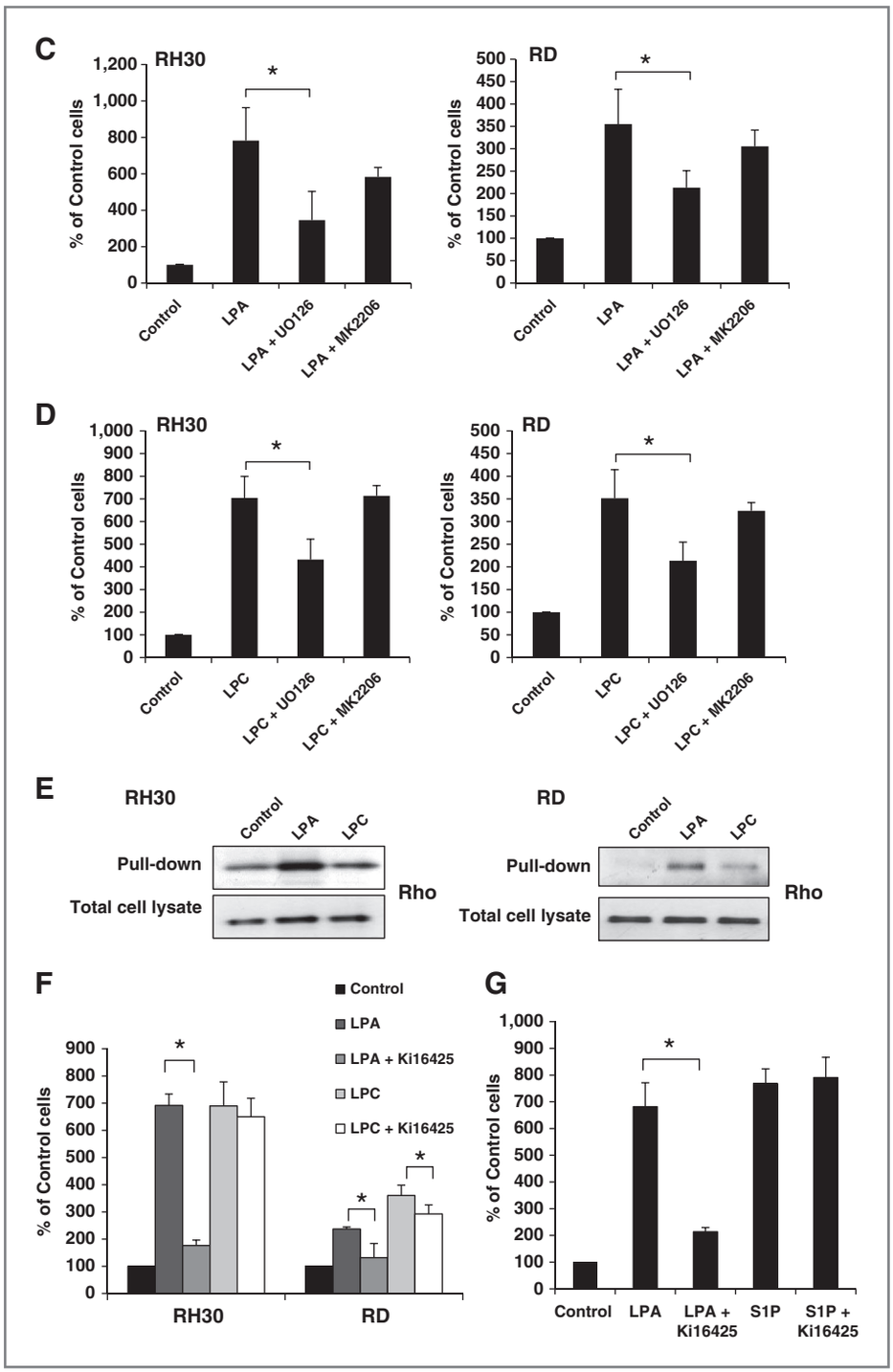

Figure 2. (*Continued*.) C, the effect of UO126 and MK2206 on the migration of RH30 (left) and RD (right) cells in response to LPA (0.1 μmol/L) The experiment was done twice with similar results. *, $P < 0.05$. D, the effect of UO126 and MK2206 on the migration of RH30 (left) and RD (right) cells in response to LPC (20 μmol/L). The experiment was done twice with similar results. *, $P < 0.05$. E, activation of Rho in human RH30 and RD cell lines stimulated for 5 minutes by LPA (0.1 μmol/L) or for 2 hours with LPC (20 μmol/L). The experiment was repeated twice, with similar results, and representative Western blots are shown. F, the effect of the LPAR1 and LPAR3 inhibitor Ki16425 (10 μmol/L) on migration of RH30 or RD cells in response to LPA (0.1 μmol/L) or LPC (20 μmol/L); *, $P < 0.05$. G, chemtoaxis of cells preatreated with Ki16425 (10 μmol/L) in response to LPA (0.1 μmol/L) and S1P (1 μmol/L) confirms specificity of the inhibitor. *, $P < 0.05$.

recovery. After peak identification and integration, the lipids of interest and recovery standards were quantitated by reference to calibration curves generated by adding a range of concentrations of the lipids of interest to an appropriate matrix (plasma or tissue lipids). The lipid standards used for these calibrations were independently quantitated by phosphorous determination or accurate mass measurements. Absolute levels of the analytes in the starting sample were then determined using these calibrations with correction for the recovery standard.

Wound-healing assay

Cells were seeded in a 6-well plate and after they reached 70% to 80% of confluence, wounds were carefully made

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across the cell monolayer, so that the surrounding cells were not disturbed. Cells were washed several times, and the medium was replaced by fresh one with 0.5% BSA with or without LPA or LPC. The cultures were monitored at several time points: 0, 24, and 48 hours. Photographs were taken under the phase contrast microscope, using a 40× magnification.

Statistical analysis

All results were presented as mean ± SD. Statistical analysis of the data was done using the Student *t* test for unpaired samples, with $P < 0.05$ considered significant.

Results

LPA and LPC strongly induce chemokinetic migration of RMS cells

First, we studied the effect of LPA and LPC on proliferation of RMS cells. However, despite reports that LPA and LPC stimulate proliferation of muscle cells (20–22), a physiologic (i.e., plasma) concentration of LPA (0.1 μmol/L) had no effect on proliferation or survival of RMS cells cultured in serum-free conditions (data not shown). Moreover, LPC was toxic to RMS cells at a physiologic (i.e., plasma) concentration (200 μmol/L), as was reported for other cells (23). However in our hands, this toxic effect was not observed at a lower concentration of LPC (20 μmol/L), which is below the known toxic threshold concentration of LPC (∼50 μmol/L). At this concentration, neither LPC nor LPA affected proliferation or survival of RMS cells cultured in serum-free conditions (data not shown). Nevertheless, because of toxicity concerns, in further experiments, we used LPC at a concentration of 20 μmol/L.

The motility of cancer cells plays a crucial role in the process of tumor metastasis, and we observed that both LPA and LPC strongly enhanced migration of RMS cells (Fig. 1). Specifically, Fig. 1A shows that both ARMS (RH30) and ERMS (RD) cells respond robustly and optimally to physiologic concentrations of both LPA and LPC. Furthermore, as demonstrated in Fig. 1B, LPA and LPC increase the motility of all eight human RMS cell lines used in our study. Moreover, Fig. 1C compares the response of the highly metastatic RH30 ARMS cell line to physiologic levels of LPA, LPC, and two other bioactive lipids, S1P and C1P, reported in our previous work to be potent chemotactic factors for RMS (10), as well as two peptide-based factors, SDF-1 (5, 6) and HGF/SF (6), used at physiologic and supraphysiologic concentrations. We found that at physiologic concentrations, both LPA and LPC, like S1P and C1P (11), increase migration of RMS cells, and the chemotactic response of RMS cells to a gradient of all these biologic lipids was much higher than observed for SDF-1 and HGF/SF at the physiologic concentrations present in peripheral blood.

To address whether the observed increase in motility of RMS cells in response to LPA and LPC is a result of a chemotactic versus a chemokinetic response, we performed a checkerboard assay in which LPA or LPC were added at the same time into the upper and the lower Transwell chambers so that no LPA and LPC gradient was created between both chambers. Figure 1D and E demonstrate that the migration of RH30 and RD cells in response to LPA in the upper and lower chambers was not significantly changed and was inhibited by approximately 30% when LPC was in both the upper and lower chambers. This finding indicates that LPA and LPC, like S1P and C1P (11), are mainly chemokinetic rather than chemotactic factors for RMS cells. The effect of LPA and LPC on cell migration was additionally confirmed by using wound-healing assay (Supplementary Fig. S1).

It is known that LPC may be converted to LPA by ATX, which was initially described as a tumor cell motility–stimulating factor (24). ATX has lysophospholipase D activity and catalyzes production of LPA in extracellular fluids. Because we observed that some RMS cells express ATX mRNA (Fig. 2A), we asked whether some of the LPC effects on RMS cell motility are dependent on conversion of an LPC to an LPA gradient. To address this question, we studied the migration of RMS cells in response to LPC in the presence or absence of the ATX small-molecule inhibitor S32826 (25). As shown in Fig. 1F, ATX inhibition reduced LPC-directed migration of RD cells and slightly affected the response of RH30 cells, which is consistent with the expression pattern of ATX in these cell lines (Fig. 2A). In a control experiment, this inhibitor did not affect migration of RMS cells in response to an LPA gradient (Fig. 1F), as expected.

Human RMS cells express functional G protein–coupled LPA and LPC receptors

Next, we used RT-PCR to assess the expression of LPA and LPC receptors in RMS cells. To address this question, cDNA was isolated from all eight RMS cells lines, and we studied the expression of LPA receptor types LPAR1–5 (15) as well as expression of receptors G2A and GPR4 that have been reported to respond to LPC (26, 27). We found that all human RMS cell lines investigated in our study expressed at least two LPA receptors known to be important for tumorigenic activity (LPAR1–3), and some of them also expressed LPAR4 and LPAR5 (Fig. 2A). Most of the cells evaluated in our study also expressed G2A and GPR4 receptors. The relative level of expression of these receptor mRNAs according to qRT-PCR is shown in Supplementary Fig. S2.

Subsequently, we used p42/44 MAPK and AKT phosphorylation studies to demonstrate the functionality of LPA and LPC receptors on RMS cells (Fig. 2B). Both signaling pathways were selected because of their well-known role in migration and adhesion of normal (28, 29) and malignant cells (30, 31). We observed that all RMS cell lines responded by phosphorylation of p42/44 MAPK in response to LPA and LPC, and AKT phosphorylation in response to LPA and LPC was more selective and occurred only in some of the ARMS cell lines. Furthermore, as demonstrated in Fig. 2C and D, although the chemokinetic responsiveness of RH30 (ARMS) and RD cells (ERMS) to LPA and LPC gradients was inhibited by UO126 (a MAPK inhibitor), inhibition by

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MK2206, a known AKT inhibitor, was ineffective, which further supports the major involvement of p42/44 MAPK in LPA- and LPC-mediated motility of RMS cells.

Because LPA activates also Rho GTPases, including RhoA, we tested whether Rho becomes activated in RH30 and RD cell lines in response to LPA and LPC stimulation. Figure 2E shows that Rho becomes activated after LPA stimulation both in RD and RH30 cells. In contrast, LPC activated Rho in ATX-positive RD cells only.

To better understand the involvement of particular LPA receptors in LPA-induced motility of RMS cells, we used the commercially available specific LPAR1 and LPAR3 inhibitor Ki16425. Figure 2F shows that Ki16425 decreased motility of RH30 and RD cells in the presence of LPA. At the same time, as expected, Ki16425 did not inhibit migration of RH30 cells to an S1P gradient, which confirms that the effect of Ki16425 is LPAR1- and LPAR3-specific (Fig. 2G). At the same time, LPA receptor 1 and 3 inhibitor (Ki16425) did not affect LPC-mediated migration of RH30 cells; however, it inhibited slightly migration of RD cell line. This could be explained by a fact that RD cells in contrast to RH30 cells express ATX, and this effect of LPC is most likely mediated by ATX-dependent conversion of LPC to LPA.

LPA and LPC increase adhesion of RMS cells

Another important feature of metastasizing cancer cells are their adhesive properties at the site of metastasis. Therefore, we next studied the effect of LPA and LPC on adhesion of RMS cell lines to fibronectin and to bone marrow–derived stromal cells. We found that both bioactive lipids strongly induced adhesion of RMS cells to fibronectin (Fig. 3A) and bone marrow–derived stroma (Fig. 3B).

The presence of LPA increases chemoresistance of cells to vincristine

To assess the role of LPA and LPC in the potential resistance of RMS cells to chemotherapy, we performed proliferation assays of RMS cells in protein-free medium supplemented with these bioactive lipids and different doses of vincristine. As demonstrated in Fig. 4A, we observed an increase in survival of RH30 cells in the presence of low concentrations of vincristine (~IC_{50}; ref. 32), and this effect was LPA concentration-dependent. A similar effect was also observed for higher concentrations of vincristine (Fig. 4B); however, the survival ratio of cells was much lower. In contrast, we did not observe a positive effect on the survival ratio of RH30 cells exposed to vincristine in the presence of LPC (data not shown). Furthermore, FACS analysis of

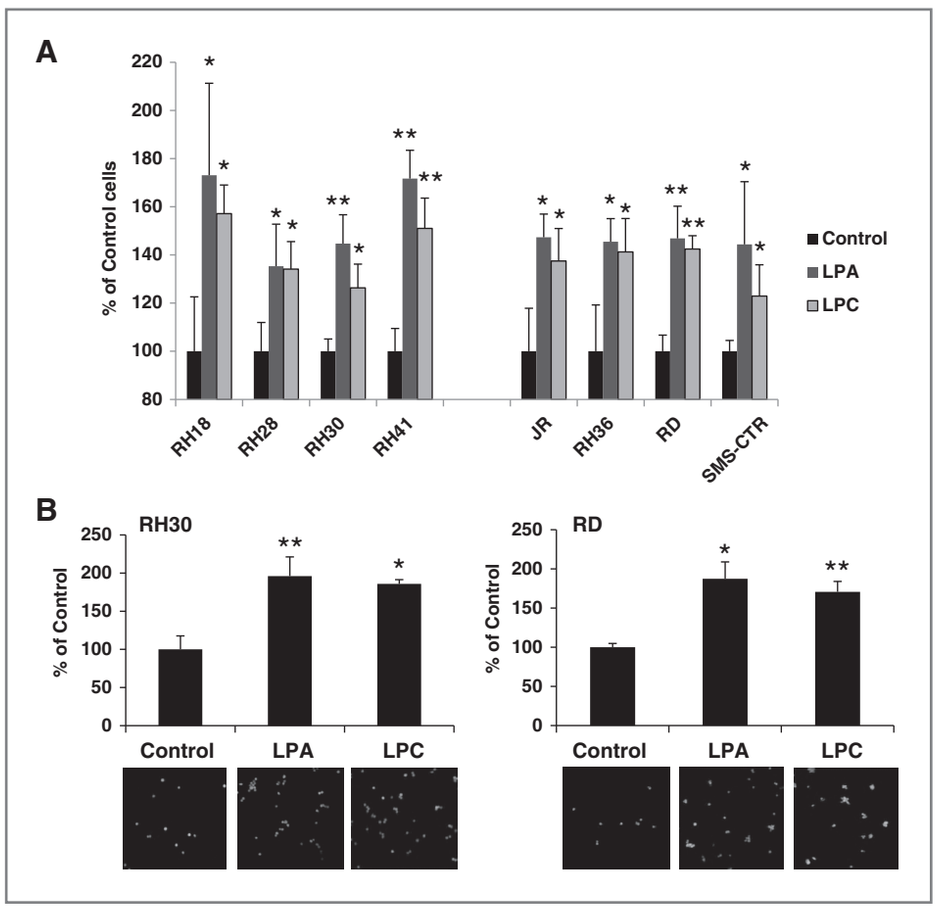

Figure 3. LPA and LPC increase the adhesivness of RMS cells. A, adhesion of RMS cells to fibronectin. The cells were not stimulated (control) or were stimulated with LPA (0.1 μmol/L) or LPC (20 μmol/L) for 10 minutes. The number of adherent cells was measured by microscopic analysis. Data from two separate experiments are pooled together and mean ± SD are shown. *, $P < 0.05$ and **, $P < 0.01$ compared with the control. B, adhesion of RH30 and RD cells to mouse stromal cells. RMS cells stained with calcein AM were stimulated with LPA (0.1 μmol/L) or LPC (20 μmol/L) for 10 minutes. After a 15-minute incubation, nonadherent cells were removed and adherent cells counted under a fluorescence microscope. *, $P < 0.05$ and **, $P < 0.01$.

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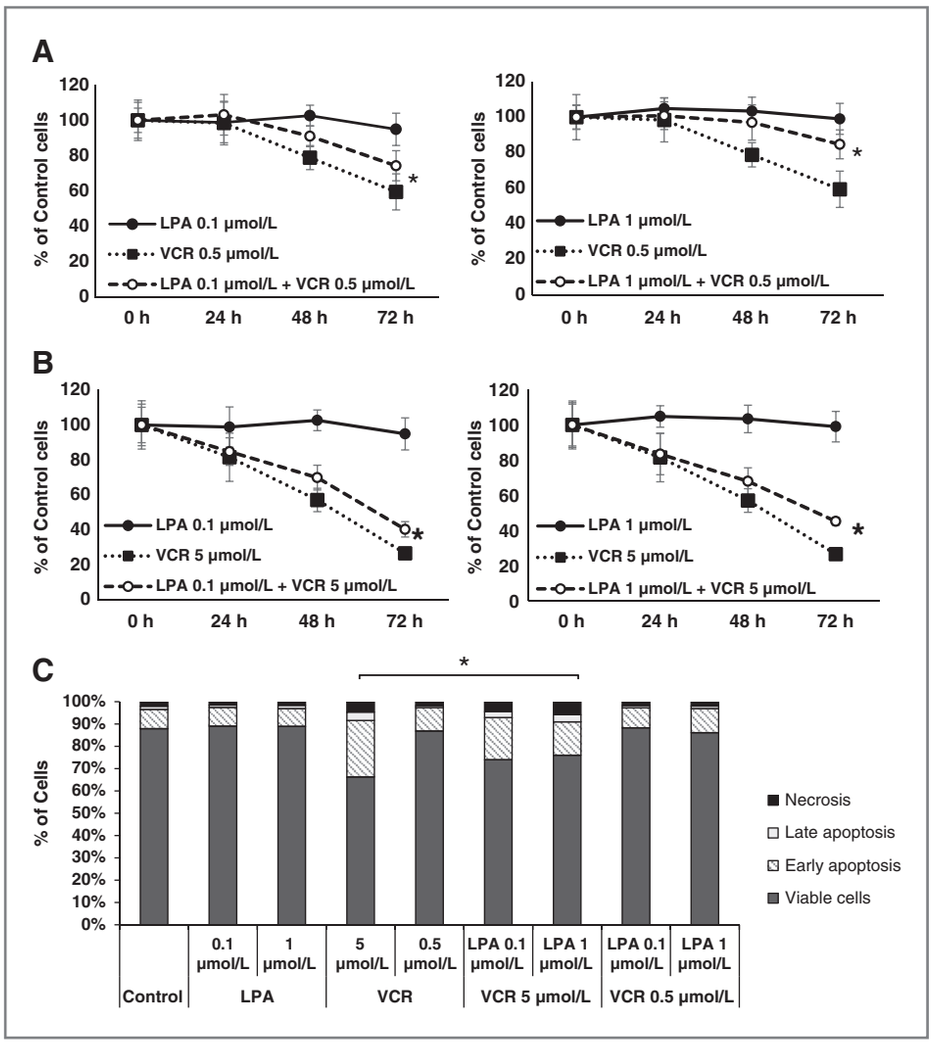

Figure 4. LPA increases the survival ratio of RMS cells treated with vincristine (VCR). A, proliferation of RMS cells in medium with 0.5% BSA in the presence of a low concentration of vincristine (0.5 μmol/L) and in the presence of two different concentrations of LPA (0.1 μmol/L, left or 1 μmol/L, right). Data from two separate experiments performed in triplicate are pooled together, and mean ± SD are shown. *, $P < 0.05$ compared with the control. B, proliferation of RMS cells in medium with 0.5% BSA in the presence of a high concentration of vincristine (5 μmol/L) and in the presence of two concentrations of LPA (0.1 μmol/L, left or 1 μmol/L, right). Data from two separate experiments performed in triplicate are pooled together, and mean ± SD are shown. *, $P < 0.05$ compared with the control. C, results of FACS analysis of Annexin V and propidium iodide staining of cells incubated for 72 hours in medium with 0.5% BSA in the presence of vincristine (0.5 or 5 μmol/L) and/or LPA (0.1 or 1 μmol/L). Data from two separate experiments are pooled together. *, $P < 0.05$.

Annexin V and propidium iodide staining of RH30 cells indicated that LPA might decrease the ratio of cells entering apoptosis (Fig. 4C), which further supports its effect on mediating the resistance of RMS cells to vincristine.

Irradiation and chemotherapy increase LPA and LPC levels in bone marrow

We have proposed that one of the unwanted side effects of radio/chemotherapy is induction of a prometastatic environment in different tissues (11, 12). To see whether radio/chemotherapy could increase LPA and LPC levels in organs affected by systemic treatment, LPA and LPC levels were measured in supernatants harvested from murine bone marrow, liver, lungs, and brain, which are frequent sites of RMS metastasis before or after exposure to irradiation or vincristine administration, by using a sensitive mass spectrometry–based approach. We observed, however, that although the concentrations of LPA and LPC in peripheral blood plasma did not change in either condition (Fig. 5A, top plots), LPA and LPC levels in bone marrow cell extracts (Fig. 5A, middle plots) as well as in conditioned medium from bone marrow cells (Fig. 5A, bottom plots) significantly increased. This finding supports the presence of a bone marrow gradient promoting motility of RMS cells toward the bones.

To address this issue better, we used conditioned medium harvested from irradiated bone marrow as a source of chemoattractants in Transwell migration assays and observed an increase in motility of RH30 cells (Fig. 5B). To address the involvement of LPA in this effect, we pretreated cells before the migration assay by incubation with Kil16425, which is an LPA receptor inhibitor, and observed a significant decrease in migration of RH30 cells to conditioned medium from irradiated bone marrow (Fig. 5B). The fact that this inhibition is not complete since the number of migrated cells is still higher than for control conditioned medium, is explained by a presence of other chemoattractants present in conditioned medium from irradiated bone marrow including, as we reported recently another bioactive lipid—S1P (11).

Of note, in addition to bone marrow, we also observed a small increase in LPA and LPC levels in other analyzed organs after exposure to radio/chemotherapy (data not shown).

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Pretreatment of cells with Ki16425 decreases LPA-dependent metastatic spread of RMS cells

Finally, we tested whether exposure of LPA receptors on RH30 cells to the inhibitor Ki16425 affects the metastatic spread of RMS cells *in vivo* to tissues damaged by irradiation by evaluating the seeding efficiency of these cells to different organs (bone marrow, lungs, and liver). To address this issue, RH30 cells were exposed to Ki16425 and injected into nonirradiated control and 750-cGy–irradiated SCID/beige mice (Fig. 5C). We observed that irradiation increases the seeding efficiency of RH30 cells, especially to bone marrow, and that this effect was significantly reduced after pretreatment of RH30 cells with Ki16425, which corroborates the observation that the LPA level is highly elevated in irradiated bone marrow (Fig. 5B). Interestingly, we also observed a significant reduction in bone marrow seeding efficiency by RH30 cells pretreated with Ki16425, which suggests that LPA is one of the major factors directing RMS cells to bone. In control experiments, the toxicity of K116425 against RMS cells was excluded in cell survival and proliferation assays (Supplementary Fig. S3A–S3F). Moreover, on the basis of one-hour exposure of RMS cells to Ki16425, followed by washed out step and delayed migration assay, we noticed that this inhibitor significantly affected responsiveness of RMS cells to LPA for at least 36 hours (Supplementary Fig. S3G).

Discussion

The salient observation of our work is that LPA and LPC are novel, potent, prometastatic factors for human RMS cells. We demonstrated the presence of both functional receptors for these bioactive lipids on RMS cells as well as their involvement in RMS cell metastasis in a set of *in vitro* and *in vivo* experiments. These observations should prompt the development of inhibitors of LPA and LPC signaling (or molecules that bind these bioactive lipids in biologic fluids and prevent their signaling through binding to the corresponding receptors) that are efficient and safe for *in vivo* administration.

It is well known that the recurrence of tumor growth after successful initial treatment and the fatal tendency of cancerous cells to spread and metastasize to different vital organs are major problems affecting the survival of patients with cancer. The ability to metastasize is one characteristic of highly malignant and primitive tumors, including RMS (2). The tropism of cancer cells to metastasize to selected organs pinpoints the involvement of organ-specific factors that direct metastasis. These factors may promote the formation of a premetastatic niche that provides metastasizing tumor cells with a favorable growth and survival environment (33).

In our previous work, we have demonstrated an important role for the α-chemokine SDF-1 in the metastasis of RMS cells to bone marrow, which is one of the common metastatic sites for RMS cells (2). We and others also reported that the metastasis of RMS cells is directed by several growth factors, including HGF/SF (6), IGFI (7), interferon-inducible T-cell alpha chemoattractant (I-TAC; ref. 17), and macrophage migration inhibitory factor (34). Moreover, in addition to these prometastatic, peptide-based factors, evidence has accumulated that a family of bioactive lipids plays an important and underappreciated role in tumor metastasis, and we recently demonstrated for the first time the involvement of S1P and C1P in the metastasis of RMS cells (11).

Because S1P and C1P are not the only bioactive lipids involved in cancer metastasis, we became interested in the role of LPC and LPA, which have both been demonstrated to be involved in metastasis, including lung, breast, prostate, ovarian, and pancreatic cancer cells (35–39). Both of these bioactive lipids are also involved in angiogenesis (40, 41) and modulate several biologic activities of normal and malignant hematopoietic cells (42–45). Nevertheless, in contrast to other reports with other types of cancer cells, in our hands, LPA and LPC did not promote proliferation of RMS cells. However, we observed that LPA promoted resistance of RMS cells to chemotherapy, as seen in an inhibitory effect of this bioactive lipid on vincristine-induced apoptosis of RH30 cells, which might explain the unwanted survival of some RMS cells after chemotherapy. Interestingly, a positive effect of LPA on chemotherapy resistance was recently reported also for breast cancer cells treated with taxol (46).

Overall, bioactive lipid signaling is thought to be qualitatively different from other classical signaling pathways, because these molecules cannot circulate freely in solution but rather exist or bound to predominantly serum albumin or associated with lipoproteins in plasma (47–49). However, their level in biologic fluids may also increase locally and acutely due to their release from damaged cells—as we have demonstrated in the case of S1P and C1P after radio/chemotherapy (11). Here, we described a similar phenomenon for LPC and LPA. Thus, the overall level of bioactive lipids in biologic fluids may increase in response to tissue/organ injury, which suggests a possible contribution of these molecules to creating a prometastatic environment in response to radio/chemotherapy (Fig. 5D).

Tumor metastasis is a multistep process, and in the first step, cells endowed with a higher motility potential detach from the primary tumor mass and migrate into the peripheral blood, lymph vessels, or internal body cavities. In this process, we distinguish two major types of cell motility: chemotaxis (directed migration to the gradient) and chemokinesis (random migration of cells in response to a chemoattractant). Although the first type of motility may explain the tropism of cancer cells to a particular organ that is a source of specific chemoattractant, the other type of motility reflects the ability of tumor cells to detach from the primary tumor in search of a new environment where they can grow and expand. These processes are tightly connected and together result in metastatic tumor growth.

In the current work, by using a checkerboard migration assay and wound-healing assay, we demonstrated for the first time that LPC and LPA induce random chemokinetic migration of RMS cells in a similar manner as we reported for the bioactive phospholipids S1P and C1P. It would be interesting to see whether this type of motility is also involved in LPA- and LPC-mediated metastasis of other types of cancer cells (e.g., lung, breast, or prostate) and

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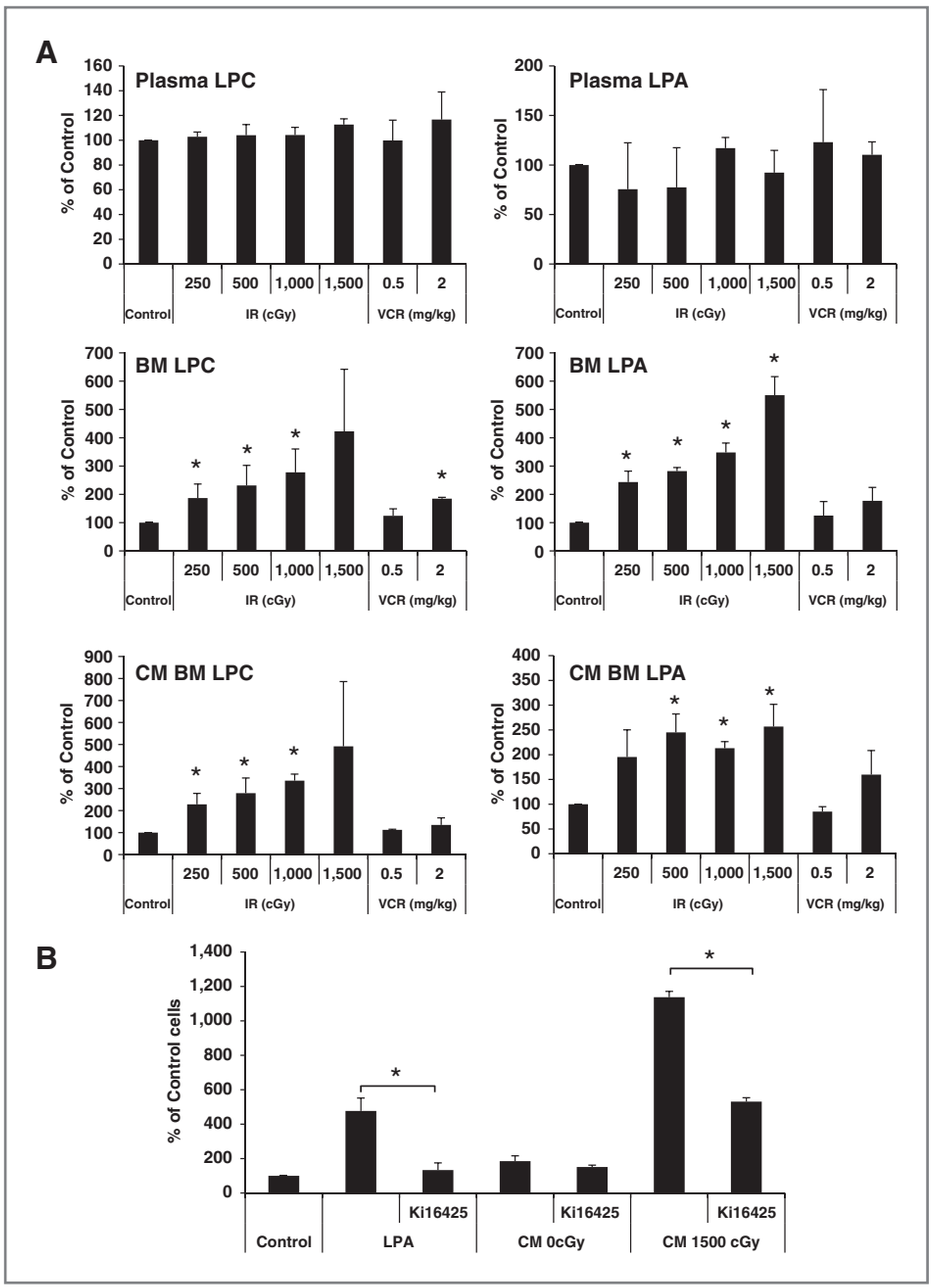

Figure 5. LPA and LPC levels create a prometastatic microenvironment in irradiated organs. A, LPC and LPA levels in plasma, bone marrow (BM), and conditioned medium (CM) from bone marrow after irradiation. Concentration of LPA and LPC in control samples were as followed: for plasma—6.72 ± 5.48 pmol/μL and 1,992 ± 1,715 pmol/μL of LPA and LPC, respectively; for bone marrow extracts—102.1 ± 137 pmol/mln of bone marrow cells and 3,677.412 ± 324 pmol/mln of bone marrow cells for LPA and LPC, respectively; for conditioned medium from bone marrow cells—0.01 ± 0.00001 pmol/μL/mln of bone marrow cells and 4.81 ± 4.18 pmol/μL/mln of bone marrow cells for LPA and LPC, respectively. B, conditioned medium from irradiated bone marrow enhances migration of RH30 cell lines across Transwell membranes, and the response of cells is attenuated in the presence of the LPAR1/3 inhibitor Ki16425 (10 μmol/L). Chemotaxis of cells in response to LPA (0.1 μmol/L) preatreated with inhibitor or vehicle only serves as controls. The results from two independent experiments are shown as mean ± SD. *, $P < 0.05$(*Continued on the following page*.)

whether it also regulates migration of normal cells, including hematopoietic, endothelial, and mesenchymal cells.

One of the most important observations in this current work is that RMS cells respond to LPA and LPC at concentrations normally encountered in peripheral blood and lymph. Thus, we can envision a scenario in which LPA and LPC are involved in increasing the overall motility of RMS cells and promote their egress from the primary tumor, whereas other factors, such as SDF-1, HGF/SF, or IGFI, tune and direct their final migration to distant organs/tissues that secrete high levels of these peptide-based chemoattractants (5).

As mentioned above, the basic clinical problem is the recurrence of metastatic tumors after radio/chemotherapy, due to the presence of therapy-resistant tumor cells that survive at the primary tumor site or in already established sites of micro-metastases. On the other hand, as we have postulated, radio/chemotherapy delivers a toxic insult to the tissues that may result in induction of a prometastatic microenvironment. In support of this notion, we have already shown not only that two important RMS prometastatic factors, SDF-1 and HGF/SF, are upregulated in bone marrow after irradiation or exposure to chemotherapy, but we have also recently demonstrated that the same is true for

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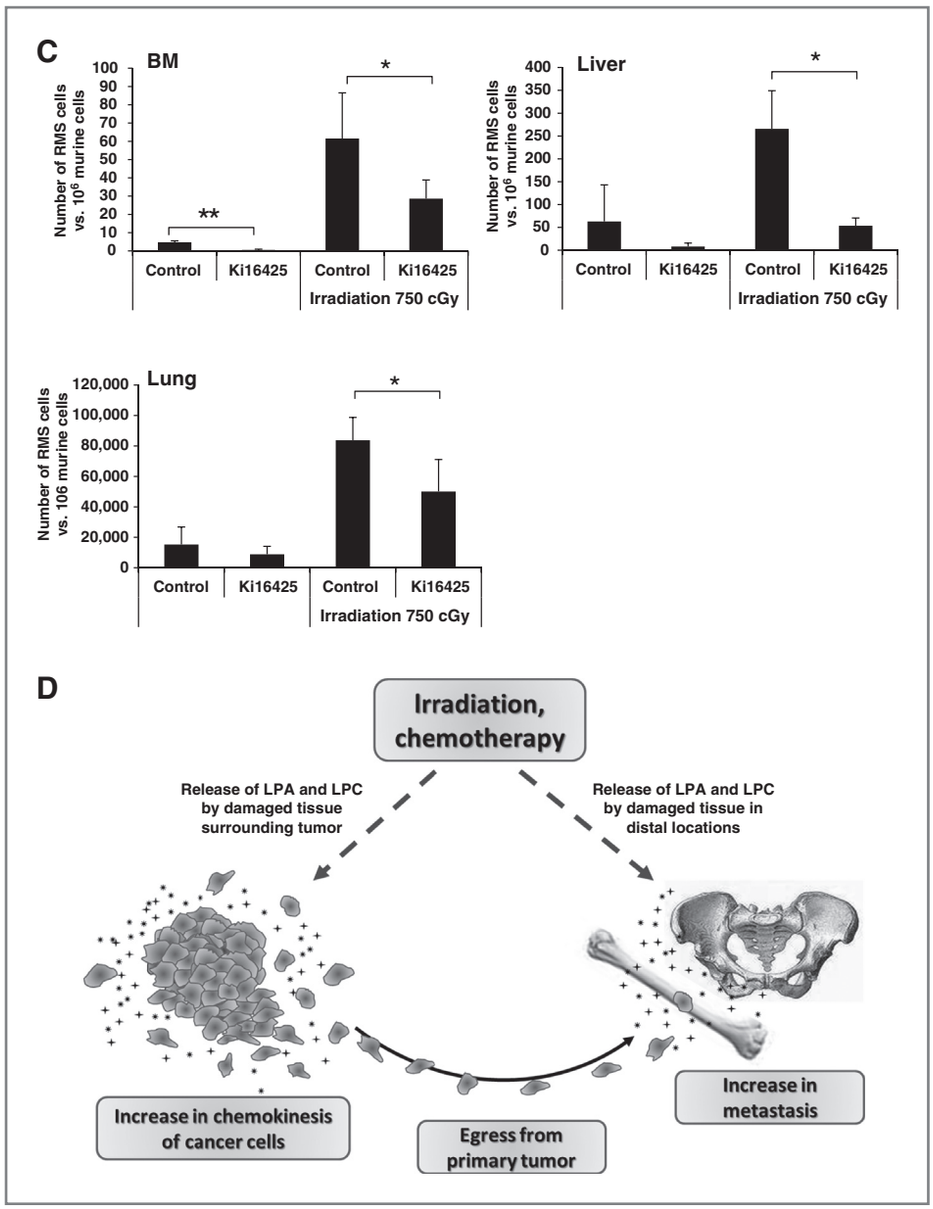

Figure 5. (*Continued*.) . C, detection of human RMS cells in organs of mice after irradiation. Before transplant, RMS cells were pretreated with Ki16425 or vehicle. In the experiment, five mice were used per group. The results are shown as mean ± SD. *, $P < 0.05$ and **, $P < 0.01$. D, schematic of the effect of LPA and LPC together with irradiation/chemotherapy on the metastasis of RMS cells from the primary tumor to bone. LPA and LPC are upregulated in response to radio/chemotherapy both in tumor surrounding and in distant tissues. As result of this, tumor cells that survive treatment become more mobile, egress primary tumor, and may seed into remote organs (e.g., bone marrow).

S1P and C1P. In this current report, we show that radio/chemotherapy may also induce an increase in LPC and LPA levels in the bone marrow microenvironment as well as in other tissues. Thus, the induction of a prometastatic microenvironment in peripheral tissues may create permissive conditions for tumor cells that survive treatment to lodge and expand, and LPA and LPC could play an important role in this phenomenon. This notion is supported by our experiments showing an LPA-mediated effect on *in vitro* migration and *in vivo* metastatic spread of RMS cells to irradiated tissues. The most visible effect we observed was for bone marrow, and it is known RMS cells, in particular ARMS cells, have a significant metastatic tropism to the bone marrow microenvironment. These findings support the conclusion that bioactive lipids are important factors that may promote homing of hematopoietic stem cells to bone marrow (42, 50) as well as promote metastasis of cancer cells to bone. The positive effect of LPA on survival of the RMS cells exposed to vincristine demonstrated in this work also explains the potential involvement of this lipid in the resistance of RMS cells to therapy.

In summary, our data for the first time demonstrate that LPC and LPA, already present at physiologic concentrations in peripheral blood or lymph, induce prometastatic behavior in RMS cells. We also demonstrate that both of these bioactive lipids become upregulated in tissues exposed to radio/chemotherapy and thus contribute to a prometastatic microenvironment in several organs, including bone marrow. Moreover, we demonstrate that the spread of RMS cells can be efficiently inhibited *in vivo* by blocking LPA

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receptors. On the basis of this finding, LPA- and LPC-mediated signaling are novel targets for developing more efficient treatment modalities for RMS and should be considered as a follow-up step to radio/chemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Abdel-Latif, A.J. Morris, M.Z. Ratajczak
Development of methodology: G. Schneider
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z.P. Sellers, A. Abdel-Latif, A.J. Morris
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Schneider, A. Abdel-Latif
Writing, review, and/or revision of the manuscript: G. Schneider, M.Z. Ratajczak
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.J. Morris
Study supervision: A.J. Morris, M.Z. Ratajczak
Other (performing experiments): G. Schneider

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Bioactive Lipids, LPC and LPA, Are Novel Prometastatic Factors and Their Tissue Levels Increase in Response to Radio/Chemotherapy

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