Preclinical Development

Lysophosphatidylcholine Pretreatment Reduces VLA-4 and P-Selectin–Mediated B16.F10 Melanoma Cell Adhesion In vitro and Inhibits Metastasis-Like Lung Invasion In vivo

Peter Jantscheff1, Martin Schlesinger2, Juliane Fritzsche2, Lenka A. Taylor1, Ralph Graeser2, Gregor Kirfel4, Dieter O. Fürst4, Ulrich Massing1, and Gerd Bendas2

Abstract

Lysophosphatidylcholine (LysoPC) is an important intermediate in degradation and biosynthesis of phosphatidylcholine (PC). Reduced plasma LysoPC levels observed in patients with advanced cancer indicate a deregulation of LysoPC metabolism in metastasis. Recent data showed strong antimetastatic effects of liposomes consisting of saturated PC in a murine pancreatic metastasis model. LysoPC, generated from saturated PC after accumulation of the liposomes in tumor tissue, might be contributing to these effects. Examining effects of high local concentrations of saturated LysoPC and investigating potential molecular mechanisms, fast removal of saturated LysoPC from medium by murine B16.F10 melanoma cells and radical shifts in tumor cell membrane fatty acid (FA) composition toward saturated FAs were observed in vitro. Scanning electron microscopy revealed remarkable morphologic surface changes of LysoPC-treated tumor cells, probably causing their impaired migratory ability on fibronectin. A LysoPC concentration exceeding a threshold of about 400 μmol/L, slightly above physiologic levels, strongly reduced VLA-4–mediated binding of B16.F10 cells to VCAM-1 as well as P-selectin–dependent interaction with activated platelets, although expression levels were not altered. These findings were reflected in a syngenic intravenous lung invasion model using repeatedly ex vivo LysoPC-treated (450 μmol/L) B16.F10 cells, resulting in significantly reduced lung metastasis-like lesions (−48.3%, \( P = 0.006 \)). Prior application of 50 IU unfractionated heparin further reduced lung invasion (−81.6%, \( P = 0.043 \)). Our work shows for the first time that saturated LysoPC in high concentrations reduces melanoma cell adhesion in vitro and hematogeneous dissemination in vivo by direct ex vivo tumor cell targeting.

Mol Cancer Ther; 10(1); 186–97. ©2011 AACR.

Introduction

Metastatic spread of solid tumors is a leading cause of death in the course of malignant diseases (1, 2). The mechanisms of metastasis, such as tumor cell invasion into the blood system, interaction with blood components, adhesion to endothelial cells, and extravasation are highly complex and not yet completely understood (3–7). Consequently, there is at present no therapeutic approach to interfere with metastatic spreading of tumors.

Recently, we observed a pronounced antimetastatic effect of empty liposomes consisting mainly of hydrogenated phosphatidylcholine in an orthotopic human AsPC1 pancreatic cancer nude mouse model (8). Because these phospholipids (PL), in contrast to physiologically occurring PL, contained only saturated fatty acids (FA), we postulated that hydrogenated PL are pivotal for this surprising effect (8).

Physiologically, plasma PLs are mostly associated with lipoproteins, surrounding the lipophilic inner core of triglycerides and cholesterol esters (9), and contain prominent amounts of unsaturated FAs (10, 11). Cells may access these PL when hydrolysis of one FA residue occurs, generating lysophospholipids (LysoPL), which can easily be taken up by the cells. PLs are hydrolyzed via enzymatic activity of either type II phospholipases A2 (PLA2; ref. 12), lecithin-cholesterol acyltransferase (LCAT; PLA2 and PLA1 activity; ref. 13), or endothelial lipases (EL; PLA1 activity; refs. 14, 15). These enzymes mostly hydrolyze lipoprotein-associated PL, both at the
SN₁ as well as the SN₂ position (11–16), thereby contributing to a pool of LysoPL that represents about one tenth of the total amount of plasma PL (9). The concentration of LysoPC was found to be constant in blood plasma of healthy persons, ranging from 200 to 300 μmol/L (16) or 300 to 400 μmol/L (17, 18), suggesting a sensitive balance between generation and removal of LysoPC (e.g., by cellular uptake). The FA composition of plasma LysoPC in healthy individuals is comparable with that of total plasma PL; our unpublished studies confirm that more than 31% of the plasma LysoPC molecules carry unsaturated FAs. Various clinical observations have shown that plasma LysoPC levels are often decreased in advanced metastatic cancer patients (17–20), especially when weight loss and inflammatory processes occur (21). This finding suggests a deregulation of the aforementioned LysoPC balance by malignant tumors and clearly indicates the potential importance of LysoPC in metastatic and cachectic processes.

The antimitastic effects of empty liposomes, as observed in the AsPC1 pancreatic cancer model (8), are supposedly a consequence of passive accumulation of liposomes in tumor tissue (8, 22, 23) due to the enhanced permeability and retention effect (24). We postulated an enzymatic hydrolysis of accumulated liposomes similar to PL in lipoproteins (12–15), such as by PLA2-active tumor cells. The resulting high intratumoral concentrations of LysoPC, containing saturated FA might be responsible for the observed effects. It is well known that PLs or LysoPLs play an important role in several biological pathways such as cellular differentiation, proliferation, migration, extracellular matrix deposition, and morphologic changes in various target cells (25–31). However, the above-mentioned liposomal study did not clearly show whether the antimitastic effect of liposomal PLs/LysoPLs results from their interaction with the tumor cells themselves and/or with other cells involved in metastatic processes such as endothelial cells, smooth muscle cells, or macrophages.

Therefore, this first study on this novel perspective intended to (i) investigate the effects of exogenous LysoPC containing saturated FAs on tumor cells in vitro and (ii) analyze its influence on the metastatic behavior of tumor cells in vivo after submission to ex vivo pretreatment.

Using the highly metastatic murine melanoma cell line B16.F10 (22–35), incubated with LysoPC containing saturated FA residues (8), a striking uptake and membrane integration of the FAs of LysoPC into the melanoma cells was observed in vitro. This treatment also reduced the in vitro interaction of B16.F10 cells with platelets via P-selectin as well as the binding of tumor cell integrin VLA-4 to VCAM-1 (34). Both are critical factors for the establishment of contact with the endothelium and therefore pivotal for metastatic activity of tumor cells (32, 34).

Analyzing the impact of LysoPC treatment on metastatic activity of the tumor cells in vivo by using an experimental murine metastasis-like lung invasion model (32–34) resulted in significantly reduced lung metastasis-like lesions from ex vivo treated melanoma cells. This confirmed that the tumor cells themselves are directly targeted by LysoPC, and their metastasis-like behavior in mice is modified as a consequence.

Material and Methods

Antibodies and adhesion molecules

Recombinant mouse VCAM-1 Fc-chimera, antimouse CD49d monoclonal antibody (mAb; integrin-α₅-chain), and recombinant mouse P-selectin Fc-chimera were purchased from R&D Systems GmbH. Anti-human IgG (Fc-specific)-FITC and human fibronectin were obtained from Sigma-Aldrich Chemie GmbH.

Cell culture and LysoPC feeding

Mouse melanoma cell line B16.F10 (American Type Culture Collection, CRL-6475) was passaged in vitro in DMEM as described before (34). Cells were routinely (monthly) tested for mycoplasmas, their morphologic appearance observed by microscopic means, and examined for the expression of adhesion receptors and ligands by flow cytometry. LysoPC removal (1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (C17:0-LysoPC; Avanti Polar Lipids, Inc.) or 1-hexadecanoyl-sn-glycero-3-phosphocholine (C16:0-LysoPC; Sigma) was determined using 1 × 10⁵ cells in 24-well tissue culture plates (Greiner BioOne). Noncytotoxic LysoPC/BSA DMEM solutions (Supplementary Fig. S1A) were prepared by adding 20–40 mg/mL of bovine serum albumin (BSA; PAA Laboratories GmbH). Cell culture supernatants were collected from triplicates of LysoPC-treated and untreated control wells. Cells were removed from tissue culture plates by using trypsin/EDTA. Centrifuged supernatants and cell pellets were stored at −20°C and −80°C until analysis, respectively.

Long-term treatments of cells were done as mentioned earlier, adding and removing cells, LysoPC, and supernatants as specified in the Results section. Cells for in vivo application were cultivated in 250-mL culture flasks either with 15 mL of 450 or 300 μmol/L of LysoPC or with DMEM-BSA culture medium as the control.

Generation of luciferase-expressing B16.F10 melanoma cells

For sensitive detection of metastases in vivo in lung and other murine tissues, stably luciferase-transduced B16. F10-ELN melanoma cells were established as recently described (22).

Bromodeoxyuridine proliferation assay

Proliferation and viability were determined by bromodeoxyuridine (BrdU) and WST-1 assay (Roche Diagnostics GmbH) according to the manufacturer’s instructions.
Determination of LysoPC concentration
LysoPC concentration was determined by an enzymatic PL (PC/LysoPC) assay (Fig. 1) containing phospholipase-D and choline oxidase (mti diagnostics GmbH). For the determination of free choline, we developed a similar assay that does not contain phospholipase-D and thus is specific for free choline (see Supplementary material). Glycerol-3-phosphocholine (GlycoPC) was determined using the assay for free choline adding 1 IU/mL of sn-glycero-3-phosphocholine phosphodies-terase (Sigma). We ensured the specificity of the 3 assays by analyzing different concentrations of LysoPC, free choline, GlycerolPC, and PC (Supplementary Fig. S1B).

Determination of FA composition in cells and culture medium
Lipid extraction was carried out according to the modified method of Bligh and Dyer (36). For derivitization, the dried lipid extracts were subjected to methanol and trimethylsulfonium hydroxide and heated up to 100°C for 10 minutes (Techne Dri Block DB-3D Techne). Gas chromatographic analysis was done by an HP-5890 Series II Plus analyzer equipped with autosampler GC/SFC injector and flame ionization detector (FID) with helium as carrier gas at a flow rate of 1 mL/min. Oven temperature program started with 150°C for 3 minutes, up to 220°C with a rate of 5°C/min, 220°C for 3.5 minutes, split injection (split flow 100 mL/min, split ratio 1:100), 5-μL injection volume, injector temperature 260°C, and FID at 280°C on an Agilent DB-23 column (30 m, 0.25-mm ID, 0.25 μm). Integration of peak areas was carried out with the HP software HP-GC ChemStation (Rev.A.06.03[509]). Peak identification was accomplished with a FA standard mixture.

Platelet isolation
Platelets from blood of healthy volunteers were separated, routinely tested for viability, and activated by TRAP-14 (Bachem GmbH) as recently described (34). Isolated platelets were stored at 37°C in a water bath.

Flow cytometric analysis and interaction of B16.F10 cells with platelets
Interaction of murine recombinant P-selectin Fc-chimera, murine VCAM-1 Fc-chimera, and antimouse CD49d (integrin-α2-chain) mAb, as well as of calcein-labeled platelets with B16.F10 melanoma cells with or without LysoPC incubation, was assessed according to recently described protocols (34).

Flow chamber assay
The interaction of B16.F10 cells with VCAM-1 Fc-chimera under physiologic flow conditions was analyzed on glass slides coated with VCAM-1 Fc-chimera in a parallel plate flow chamber, as described in detail previously (34).

Quantification of cell motility
Motility of B16.F10 cells cultivated on fibronectin (Sigma-Aldrich Chemie)-covered dishes was determined with or without LysoPC. Phase-contrast image series of motile cells were obtained 48 hours later using an inverted microscope (Nikon) and an incubation chamber for constant temperature, applying NIS Elements AR-2.30 software and a camera (COOL-1300I; Vosskuhler GmbH).

Lamella dynamics were analyzed by the computer-assisted stroboscopic analysis of cell dynamics (SACED). At least 15 individual cells, preincubated for 48 hours with or without LysoPC, were then monitored over a period of 10 minutes by capturing digital images every 2 seconds. Subsequently, 8 areas of interest were marked on each image by lines that crossed the cell lamella. The resulting 1 pixel wide areas were cut and lined up in time space plots that allowed the quantification of relevant motility data (37).

Scanning electron microscopy
For scanning electron microscopy (SEM), B16.F10 cells incubated with or without LysoPC were washed with PBS and fixed with 3% paraformaldehyde/1% glutaraldehyde in PBS for 30 minutes according to Svitkina and colleagues (38). All specimens were mounted on aluminum sample holders and coated with a 2-nm layer of platinum/palladium in an HR-208 sputter-coating device. SEM was carried out with a XL-30-SFEG scanning electron microscope (Philips).

Animal experiments
Lung metastases were induced by injecting 3 × 10⁵ cells in 100 μL of PBS into the tail vein of male C57Bl/6N mice (Charles River Laboratories; ref. 32). Animals received B16.F10 cells either treated in vitro 7 × with 450 μmol/L of LysoPC (long-term) within 10 days, 2 × with 300 μmol/L of LysoPC within 2 days, or cultured in DMEM-BSA as control (identical frequency of medium renewal, cell splitting). In a further experiment, animals were treated with 50 IU (32) unfractionated heparin (Ratiopharm GmbH) 30 minutes prior to cell injection, followed by injection of either long-term 450 μmol/L of LysoPC-
treated or DMEM-BSA control medium cultured B16.F10 cells. Experiments were terminated on day 14 (see establishment of the model, Supplementary Fig. S2). Tissues were removed and stored as snap-frozen samples. Metastatic lesions were quantified by homogenizing the tissues in luciferase lysis buffer and measuring in a luciferase assay (Promega E1501) as described (23).

Statistics
 Statistical analyses were carried out using Student’s t test or, if normality test failed, using Mann–Whitney rank-sum test (SigmaStat 3.1).

Results

In vitro effects of exogenous LysoPC on B16.F10 tumor cells

Removal of exogenous LysoPC from culture medium by B16.F10 melanoma cells. Adding various concentrations of exogenous LysoPC to the culture medium, its removal by the B16.F10 melanoma cells was measured in time-course experiments. To avoid LysoPC toxicity (i.e., apoptosis or cell lysis), suitable “protecting” concentrations of BSA were added to the medium (for details, see Supplementary Fig. S1A). No relevant LysoPC concentrations were determined in BSA-medium alone (Supplementary Fig. S1C). LysoPC removal was determined by measuring total choline (LysoPC and free choline) and subtracting the values of free choline as measured separately by a specific free choline assay (for details, see Supplementary Fig. S1C). Both B16.F10 and luciferase-transduced B16.F10 cells rapidly reduced the LysoPC (450 μmol/L), whereas LysoPC concentration was unchanged in absence of cells (Fig. 2A). This striking removal of LysoPC by the tumor cells was accompanied by equimolar release of free choline, free FAs, and significant amounts of GlyceroPC into the supernatant (not shown). The percentage of LysoPC reduction was found to be independent of the supplied LysoPC concentration (150, 300, 450, or 900 μmol/L) or the LysoPC species used (C16:0-LysoPC or C17:0-LysoPC). The removal kinetics of LysoPC by B16.F10 cells were not influenced by 4× repeated (every 48 hours) addition of further exogenous LysoPC (Fig. 2B).

Cell membrane alterations induced by LysoPC treatment. To analyze membrane alterations caused by exogenous LysoPC, the kinetics of changed FA profiles of B16.F10 melanoma cells were determined by gas chromatographic (GC) analysis. Incubation of the cells with C17:0-LysoPC resulted in a rapid increase of the ratio of C17:0 FA after 24 hours (Fig. 3A). Between 48 and 72 hours, a plateau was apparently reached (C17:0 ~55%). Ratios of all other investigated FAs decreased accordingly. Preliminary results (not shown) suggest the rate of membrane alterations to be independent of the supplied LysoPC concentrations (300, 450, or 900 μmol/L). Also, 3× repeated exposure (every 48 hours) to LysoPC did not further change the membrane composition in treated B16.F10 melanoma cells (Fig. 3B). However, GC analysis did not allow the characterization of the molecular lipid structure containing the C17:0 FAs. Similar kinetics, but with higher baseline ratio, were observed using C16:0-LysoPC (data not shown).

The incorporation of the FA from exogenous LysoPC was rapid, but the altered membrane composition remained rather stable after replacing LysoPC-containing medium by LysoPC-free culture medium. After 48-hour incubation in LysoPC-free DMEM-BSA medium, the C17:0 FA ratio decreased by only 10% to 15% (Fig. 3C).

To determine potential cell surface alterations resulting from LysoPC treatment and the observed change in FA membrane composition, SEM images of B16.F10 cells were taken. While only a few finger-like protrusions were visible in untreated cells, which might be referred to as filopodial spikes, a remarkably augmented number of protrusions (quantified in Supplementary Fig. S4) was recognizable after LysoPC incubation for 3 days (once) and 20 days (9×) with 450 μmol/L of LysoPC (Fig. 3D). In
contrast, neither the short nor the long incubation with 300 µmol/L of LysoPC significantly altered the number of protrusions compared with untreated cells (Fig. 3D).

Impact of LysoPC treatment of B16.F10 cells on adhesion receptor activity in vitro. To investigate the impact of LysoPC on the receptor-mediated cell-binding ability, in relation to concentration and incubation time, VLA-4-mediated adhesion of B16.F10 cells to a support-fixed VCAM-1 layer was examined. A single incubation of cells with 450 µmol/L of LysoPC for 3 days, followed by VLA-4 activation with Mn²⁺, significantly impaired cell binding whereas a lower LysoPC concentration of 300 µmol/L did not significantly affect binding (Fig. 4A and B). The LysoPC effects on adhesion were amplified by repeated LysoPC exposure. B16.F10 cells treated 6× with 450 µmol/L of LysoPC (a total of 13 days) showed complete binding inhibition and behaved like nonactivated control cells, whereas the repeated addition of 300 µmol/L of LysoPC had no effect on VLA-4 binding reduction (Fig. 4A and B). To further analyze this unexpected difference, which was not directly reflected by the LysoPC consumption or the change of membrane FA composition, but rather by the protrusion formation, we increased the LysoPC concentrations from 300 to

Figure 3. FA composition and morphology of LysoPC-treated B16.F10 cells. A, composition of FAs (% of total FA) in cell lysates was determined by GC analysis after exposing B16.F10 cells to 450 µmol/L of C17:0-LysoPC for indicated times. C17:0 reached a plateau between 48 and 72 hours. B, further addition of exogenous C17:0-LysoPC to the cells every 48 hours (after 48, 96, and 144 hours) did not change the FA membrane composition any further. C, discontinuing the LysoPC exposure of cells with altered membrane composition (after 48 hours) by adding DMEM-BSA medium, the FA ratio was reduced only by 15% to 20% during (–) indicated time. D, cell surface alterations induced by LysoPC treatment of B16.F10 cells were determined by SEM. Images of B16.F10 cells grown on glass slides and coated with platinum/palladium were taken without LysoPC treatment (no LP) or after LysoPC incubation for 3 and 20 days, respectively, with 300 µmol/L (3 and 20 days) or 450 µmol/L of LysoPC (3 and 20 days). While only a few finger-like protrusions were visible per area in untreated and 300 µmol/L treated cells, an augmented number was present after 3- and 20-day exposure to 450 µmol/L of LysoPC (bars correspond to 20 µm within the images and to 2 µm within image sections). In contrast to the number of finger-like protrusions, their size was nearly unchanged (ranging from 0.5 to 1–5 µm) by the treatment. Data and statistics are summarized in Supplementary Fig. S4.
450 μmol/L in steps of 25 μmol/L and examined the binding properties of cells treated 6× for a total of 13 days. A threshold concentration of about 425 μmol/L of LysoPC became apparent, leading to significant inhibition of cell adhesion via VLA-4–VCAM-1 interaction (Fig. 4C).

Because VLA-4 also has extracellular matrix-binding characteristics and we found remarkably increased number of filopodial spikes in LysoPC-treated cells, an impact on migratory characteristics was assumed. Examining migration of LysoPC-treated B16.F10 cells on a fibronectin substrate by microscopic SACED cell migration assay (Fig. 5A) clearly indicates that 3-day incubation with 450 μmol/L of LysoPC already significantly reduced (P < 0.05) motility regarding lamellipodia retraction velocity in B16.F10 cells. Frequencies of lamellipodia protrusion and retraction were also significantly reduced (P < 0.05) after LysoPC treatment (Fig. 5A).

P-selectin–mediated interaction of tumor cells with platelets is pivotal for the metastatic process. Thus, we investigated whether LysoPC treatment of the B16.F10 cells also affects this adhesion and found significantly reduced (P < 0.001) P-selectin–mediated interaction with activated platelets. Three-day LysoPC treatment (450 μmol/L) of B16.F10 cells already decreased activated platelet binding to the level of nonactivated platelets (P = 0.748; Fig. 5B). Using a specific P-selectin blocking antibody versus a control antibody, the P-selectin specificity of platelet interaction in control cells could be confirmed (P < 0.05) whereas the significantly reduced binding after LysoPC treatment of B16.F10 cells was not further affected by the antibodies (P = 0.552; Fig. 5B).

Reduced expression levels of the adhesion receptors after LysoPC exposure might explain the decreased binding activities of VLA-4 and P-selectin in the LysoPC-treated cells. However, flow cytometric analysis using B16.F10 cells repeatedly incubated with 450 μmol/L of LysoPC or DMEM-BSA for 13 days showed that LysoPC treatment did not influence VLA-4 receptor expression (Fig. 5C). Although the ability of VLA-4 to interact with soluble VCAM-1 Fc-chimera was slightly impaired, it did not concur with the strongly reduced binding to fixed VCAM-1 in the flow chamber assays (Fig. 4). Furthermore, the expression of P-selectin ligands as detected by P-selectin Fc-chimera binding also remained unchanged in LysoPC-treated B16.F10 compared with control cells (Fig. 3C), which contradicts the total inhibition of cell binding to activated platelets (Fig. 5B).

**Effects of exogenous LysoPC on metastasis-like behavior of B16.F10 tumor cells in vivo**

**Inhibition of in vivo metastasis of B16.F10 cells pretreated with LysoPC.** We compared the “metastatic spread” of untreated B16.F10 cells with those treated 6× with 450 μmol/L of LysoPC in a syngenic C57Bl/6N (n = 14) intravenous lung invasion model (Fig. 5). Luciferase-transduced B16.F10 cells were used for sensitive quantification of metastatic spread into the lung. This advanced
Figure 5. Extracellular matrix–binding characteristics, P-selectin–mediated platelet interaction, and surface expression of adhesion molecules of LysoPC treated B16.F10 cells. A, effects of LysoPC incubation on migratory characteristics of B16.F10 cells on a fibronectin substrate were determined by microscopic SACED cell migration assay. For this purpose, velocities and frequencies of lamellipodia protrusion and retraction were analyzed. A 3-day incubation with 450 μmol/L of LysoPC led to significantly (*, P < 0.05) reduced retraction velocity, and reduced protrusion and retraction frequency, compared with untreated cells. B, TRAP-14 activation of calcein-labeled platelets induced a significant P-selectin–mediated interaction to B16.F10 cells compared with nonstimulated cells, which was inhibited by 3-day pretreatment with 450 μmol/L of LysoPC (***, P < 0.001) or adding P-selectin blocking (*, P < 0.05) but not control mAbs. Adding P-selectin blocking or control mAbs to LysoPC-treated B16.F10 cells confirmed the P-selectin specificity of the effect. C, surface expression (anti-VLA4) of VLA-4 determined by flow cytometric analysis using a rat anti-mouse VLA-4 mAb did not differ between B16.F10 cells treated with 450 μM of LysoPC for 13 days (LysoPC–VLA-4) and untreated cells (VLA-4). Functional binding capacity (VCAM1 Fc) of VLA-4 on LysoPC-treated B16.F10 cells, on the contrary, showed a slightly reduced binding with soluble VCAM-1 Fc-chimera. The expression and accessibility of P-Selectin ligands (P-Selectin Fc) on 450-μmol/L of LysoPC-treated B16.F10 cells, however, were not modified by the treatment, as shown using soluble P-selectin Fc-chimera.
metastasis model is described in more detail in Supplementary data (Supplementary Fig. S2).

Fourteen days after tumor cell injection, homogenized lung extracts of the B16.F10 450-μmol/L group displayed significantly reduced luciferase activity compared with the untreated control (P = 0.006). The luciferase results were clearly confirmed by macroscopically visible metastasis, as 3 representative examples of lung surfaces of each group show (Fig. 6, bottom). Remarkably smaller numbers of metastatic foci were observed in the long-term 450-μmol/L LysoPC group compared with the control group.

In addition, in the same model (n = 10), we examined cells treated twice with 300 μmol/L of C17:0-LysoPC, when no effect on the tumor cell adhesion was observed in vitro (Fig. 4). The results from this group differed neither in luciferase activity (P = 0.279) nor macroscopically from untreated controls but significantly with the 450-μmol/L group (P = 0.001; Fig. 6).

Enhanced effect of long-term LysoPC pretreatment of cells in heparinized mice. Antimetastatic effects of heparin due to different molecular targets (e.g., P- or L-selectin and VLA-4), also affected by LysoPC in our study, are well documented (32–35). To compare the effects of LysoPC pretreatment of B16.F10 cells and intravenous heparin, we analyzed metastatic spread of control and 450 μmol/L pretreated B16.F10 cells in mice heparinized with 50 IU, which comes close to the dose known to have the maximum inhibitory effect on metastatic spread in the B16.F10 model (32). As shown in Fig. 7, both LysoPC treatment and heparin injection significantly reduced metastatic invasion compared with
untreated control (51.7%, $P = 0.006$ vs. 43.6%, $P = 0.004$). No difference between LysoPC and heparin pretreatment was observed ($P = 0.603$), but, interestingly, the injection of LysoPC pretreated cells into heparinized mice caused an enhanced effect, further reducing the occurrence of metastasis to 18.4%, significantly stronger than LysoPC ($P = 0.043$) or heparin ($P = 0.019$) alone.

**Discussion**

One of the critical steps of metastasis is the hematogenous dissemination of tumor cells (32, 33, 39–42), which includes (i) the intravasation of tumor cells, (ii) their survival in the blood stream, (iii) tumor cell binding to vascular endothelial cells, and (iv) the extravasation into target tissue(s). Cellular adhesion receptors (i.e., P- and L-selectin, or VLA-4) are considered key players in the metastatic process, mediating tumor cell interaction with endothelial cells or aggregation with platelets and leukocytes (43, 44), latter resulting in tumor cell emboli in the microvasculature (33).

The present study is based on our recent observation that empty liposomes consisting of saturated PL displayed pronounced antimetastatic effects in an orthotopic human AsPC1 pancreatic cancer nude mouse model (8). We postulated that saturated LysoPC, as degradation product of empty liposomes, might have pivotal antimetastatic activity. Investigating this hypothesis, we showed for the first time that LysoPC treatment of B16.F10 cells with saturated C16:0-LysoPC or C17:0-LysoPC has a strong impact on tumor cell adhesion and motility not only in *vitro* but also on metastasis-like hematogeneous lung invasion in *vivo*.

B16.F10 melanoma cells were exemplarily selected to represent solid tumors with high metastatic capacity. Establishment and application of luciferase-transduced B16.F10 cells for these experimental metastasis assays in mice strongly increased the informational value and allowed a more sensitive analysis, compared with the merely visual evaluation of metastatic foci in the lungs (Supplementary Fig. S2). Furthermore, the B16.F10 melanoma lung invasion model has the advantage that LysoPC treatment of the tumor cells can be done *ex vivo*. This setup allowed us to investigate the effects of LysoPC specifically on the tumor cells, without risking adulteration of results by other cells involved in metastatic processes (e.g., endothelial or smooth muscle cells or macrophages) possibly also being affected by LysoPC (25–31). LysoPC has been shown to support invasiveness and metastatic capacity of tumors, such as by impairing endothelial barrier function (31), helping to maintain tumor formation via GRP4 signaling (45), or activating Rac and Rho GTPases (29), but concentrations used in these studies were clearly below our threshold amount used here.

During *in vitro* treatment, B16.F10 cells showed extremely fast, dose-independent, and unsaturable removal of exogenously added LysoPC from culture medium. We postulated that saturated LysoPC, as degradation product of empty liposomes, might have pivotal antimetastatic activity. Investigating this hypothesis, we showed for the first time that LysoPC treatment of B16.F10 cells with saturated C16:0-LysoPC or C17:0-LysoPC has a strong impact on tumor cell adhesion and motility not only *in vitro* but also on metastasis-like hematogeneous lung invasion *in vivo*.

B16.F10 melanoma cells were exemplarily selected to represent solid tumors with high metastatic capacity. Establishment and application of luciferase-transduced B16.F10 cells for these experimental metastasis assays in mice strongly increased the informational value and allowed a more sensitive analysis, compared with the merely visual evaluation of metastatic foci in the lungs (Supplementary Fig. S2). Furthermore, the B16.F10 melanoma lung invasion model has the advantage that LysoPC treatment of the tumor cells can be done *ex vivo*. This setup allowed us to investigate the effects of LysoPC specifically on the tumor cells, without risking adulteration of results by other cells involved in metastatic processes (e.g., endothelial or smooth muscle cells or macrophages) possibly also being affected by LysoPC (25–31). LysoPC has been shown to support invasiveness and metastatic capacity of tumors, such as by impairing endothelial barrier function (31), helping to maintain tumor formation via GRP4 signaling (45), or activating Rac and Rho GTPases (29), but concentrations used in these studies were clearly below our threshold amount used here.

During *in vitro* treatment, B16.F10 cells showed extremely fast, dose-independent, and unsaturable removal of exogenously added LysoPC from culture medium. This has also been observed in other tumor cells (Jantscheff et al., unpublished data) and this effect can be discussed as one possible cause for the strongly reduced LysoPC plasma amounts observed in patients with advanced metastatic cancer (17–21). In addition, a radical shift in tumor cell membrane FA composition toward saturated FAs was observed, culminating between 48 and 72 hours without increasing after further LysoPC exposure (Fig. 3).
Another consequence of LysoPC exposure was a striking change of cell surface morphology as shown by an altered number of filopodial spikes in SEM. Even though the treated cells exhibited a remarkably augmented number of protrusions, the cell motility as essential requirement for distinct steps of metastasis, such as tissue transmigration (46), nevertheless, was significantly attenuated as determined by lamellipodia retraction and protrusion velocity or dynamics (i.e., frequencies) on fibronectin (Figs. 3 and 5). The significantly affected proportion of saturated to unsaturated FAs after LysoPC exposure might be one of the molecular mechanisms responsible for these massive changes. The ratio of saturated to unsaturated FAs in membrane PL is a fundamental biophysical determinant of membrane fluidity (47). Because the homeoviscous adaptation is regulated in a complex mode, influencing important membrane properties such as flexibility, lipid raft composition, or permeability (47–50), these FA modifications might induce the observed changes in cell surface structure and receptor activity or interaction. Another molecular mechanism possibly responsible for the remarkable alterations in cellular motility is the effect of LysoPC in signaling pathways of Rho family GTPases. These GTPases are known to be essential for the regulation of the actin filaments and thus a key player in lamellipodia activity (46). LysoPC has not only been shown to inhibit RhoA activation via Gz-coupled receptors, thereby decreasing cell migration and invasion of melanoma cells in vitro (30), but also, as shown recently in our group, to reduce gene expression of Rho GTPases in LysoPC-treated human melanoma cells (51). This molecular modification might also be a reason for the discrepant finding of an augmented number of filopodial spikes but lower functional activity. Further elucidation of these findings will be the focus of future studies using gene arrays and functional analysis.

The treatment with LysoPC not only reduced the cell motility but also led to inhibition of cell adhesion via VLA-4 to solid phase VCAM-1 and of P-selectin–dependent interaction with activated platelets in vitro. The inhibitory effects of LysoPC on B16.F10 cell adhesion were observed after approximately 48 to 72 hours at a threshold concentration of 400 μmol/L or greater, slightly higher than physiologic human plasma levels (200–400 μmol/L; refs. 16–18), and were remarkably intensified by repeated application of LysoPC. Because exposure to LysoPC concentration above the threshold did not alter expression levels of VLA-4 and P-selectin or binding to soluble structures (Fig. 5C), we assume that the higher proportion of active filopodia might spatially restrict the accessibility of the receptors for cellular or support-fixed ligands (Figs. 4 and 5B).

These in vitro findings were also reflected in a syngenic intravenous lung invasion model using ex vivo treated B16.F10 cells, and repeated exposure to LysoPC concentrations above the threshold (450 μmol/L) resulted in significantly reduced metastasis-like lesions in lung tissue. In agreement with the functional in vitro data, treatment of cells twice with lower, more physiologic LysoPC concentrations (300 μmol/L) did not influence lung colonization. These results indicate that an exposure of tumor cells to LysoPC levels above a threshold concentration that is greater than normal plasma concentration might be pivotal for the antimitastatic effect. Such an exposure of tumor cells to LysoPC levels above a threshold concentration might also occur when empty liposomes are applied, as in the AsPC1 pancreatic cancer model (8). We postulate that a locally increased intratumoral LysoPC concentration might be a consequence of enzymatic hydrolysis of liposomal PLs (12–15), such as by PLA2-active tumor cells themselves, accumulated passively in the tumor tissue due to the enhanced permeability and retention effect (24).

Heparin pretreatment in former studies has been shown to exert antimetastatic activity partially via similar targets as LysoPC treatment (VLA-4, P-selectin, or L-selectin) in vitro and to also inhibit lung invasion in the B16.F10 model (32–35). Therefore, we compared the effects of the 2 substances in our in vivo model. Although both treatments resulted in comparable decreases of lung invasion, the combination of heparin and LysoPC pre-treatment resulted in an even stronger effect (Fig. 7). Therefore, further targets of LysoPC or heparin cannot be excluded. However, the present data do not allow us to clearly discriminate whether the effects of the 2 drugs are rather additive or synergistic.

In conclusion, our data for the first time provide evidence that the exposure of B16.F10 melanoma cells to threshold concentrations of exogenous LysoPC with hydrogenated C16:0-FA or C17:0-FA residues significantly reduces their ability to metastasize in a lung invasion model. These results offer a completely new insight into LysoPC metabolism of tumor cells with possible consequences for cancer treatment. Further studies are highly warranted to gain deeper insight into the molecular mechanisms of the antimitastic activities of LysoPC and the striking additive or synergistic effect in combination with heparin that promises progress in the development of therapeutic strategies against metastatic malignancies.

Disclosure of Potential Conflicts of Interest

U. Massing, P. Jantscheff, G. Bendas, and M. Schlesinger are owners of European patent application 10156776.6-2123 (17.03.2010): “Combined Use of Phospholipids and Sulphate Groups Carrying Polysaccharides for Inhibition of Metastatic Spread.”

Grant Support

This work was supported by a grant of the Phospholipid Research Center Heidelberg to P. Jantscheff and by Deutsche Forschungsgemeinschaft (DFG: GBK 677) supplying a stipend to M. Schlesinger.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 26, 2010; revised October 19, 2010; accepted October 28, 2010; published online January 10, 2011.

Lysophosphatidylcholine, Adhesion, and Lung Invasion

Mol Cancer Ther; 10(1) January 2011


47. Mansilla MC, Banchio CE, de Mendoza D. Signalling pathways con-


Lysophosphatidylcholine Pretreatment Reduces VLA-4 and P-Selectin–Mediated B16.F10 Melanoma Cell Adhesion In vitro and Inhibits Metastasis-Like Lung Invasion In vivo

Peter Jantscheff, Martin Schlesinger, Juliane Fritzche, et al.