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Lipid profile of platelets and platelet-derived microparticles in ovarian cancer

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ABSTRACT

Background: Ovarian cancer patients have a high risk of developing venous thrombosis. The membrane lipid bilayer of platelets and platelet-derived microparticles (PMP) provides a platform for assembly of coagulation proteins and generation of blood clots.
Methods: We compared the lipid composition of platelets and PMPs in patients with ovarian cancer to those in healthy subjects. We used shotgun lipidomics to quantify 12 classes and 177 species of lipids.
Results: We found a significant change in 2 classes of lipids in platelets and PMPs isolated from ovarian cancer patients: higher phosphatidylinositol and lower lyso-phosphatidylcholine. The level of 28 species of lipids was also significantly altered in the direction of an increase in the pro-coagulant and a reduction in the anticoagulant lipids. We found that cancer platelets expressed less lipid phosphate phosphatase 1 (LPP1), a key enzyme in phospholipid biosynthesis pathways, than normal platelets. The reduction in LPP1 might contribute to the changes in the lipid profile of cancer platelets.
Conclusion: Our results support a procoagulant lipid profile of platelets in ovarian cancer patients that can play a role in the increased risk of venous thrombosis in these patients.
General significance: As far as we are aware, our study is the first study on platelet lipidomics in ovarian cancer. The importance of our findings for the future studies are: 1) a similar change in lipid profile of platelets and PMP may be responsible for hypercoagulability in other cancers, and 2) plasma level of high-risk lipids for venous thrombosis may be useful biomarkers.

1. Introduction

Hypercoagulability, an activated state of coagulation that can be detected in a majority of cancer patients [6,17,28], can remain as a subclinical condition or results in thrombosis. Venous thromboembolism (VTE) is a challenging complication of malignancy and the second leading cause of death in cancer patients. Several studies have identified a variety of risk factors for VTE [9], however, the exact molecular mechanisms for the cancer-associated hypercoagulable state and venous thrombosis remain unknown.

Coagulation is the final result of a close interaction between coagulation cascade and platelet membrane. Membrane lipids of activated platelets, particularly phosphatidylserine (PS), mediate binding and assembly of coagulation factors, which in turn promote generation of thrombin and fibrin. Other lipids in the platelet membrane interact with PS, and have regulatory roles as either procoagulant or anticoagulant lipids [24]. Phosphatidylinositol (PI), phosphatidic acid (PA), and phosphatidylglycerol (PG) were demonstrated to be procoagulants; while phosphatidylcholine (PC), acyl-carnitines (CAR) [4], and sphingomyelins (SM) [12] are anticoagulants. The effect of the other lipid classes on coagulation is not clear. The role of ceramides (CER) in platelet function is uncertain [16]. Cardiolipins (CL) are almost exclusively present in mitochondria [5]. The net effect of phosphatidylethanolamines (PE) on coagulation is hard to assess because initially PE synergizes with PS to promote coagulation, but later inactivates FVa [19,20]. Lyso-PE (LPE) have not been reported to be involved in coagulation. There are controversial reports on the role of LPCs in coagulation [25].

Each of these lipid classes includes several lipid species. Lipid species within a lipid class have similar structural features and usually similar functional roles in coagulation [24,25]. Activated platelets also release

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small vesicles encircled by a lipid bilayer, known as platelet-derived microparticles (PMP), that are highly procoagulant and can initiate and propagate coagulation [22]. In cancer patients, the crosstalk between platelets and cancer cells results in platelet activation and in a repertoire of molecular changes in platelets and cancer cells [2]. We speculated that cancer-related changes in platelet composition of molecules, and consequently in PMPs, might contribute to a hypercoagulable state and increased risk of thrombosis in cancer patients. We compared the lipid content of platelets and PMPs in patients with ovarian cancer to that in healthy controls. We identified alterations in lipid content of platelets and PMPs in cancer patients at both lipid class and species levels, with a majority of changes in the direction of promoting coagulation. Furthermore, we found that microparticle generation by cancer patients' and healthy subjects' platelets result in a similar pattern of enrichment of the lipids content in PMPs.

2. Materials and methods

All of the studies were approved by the Institutional Review Board of The University of Texas M. D. Anderson Cancer Center and in accordance with an assurance approved by the US Department of Health and Human Services.

2.1. Reagents

Chloroform, methanol, and isopropanol were purchased from Burdick and Jackson (Muskegon, MI). Lithium chloride and lithium hydroxide were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). All of the lipid internal standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) unless otherwise indicated. 1,2-Dimyristoleoyl-*sn*-glycero-3-phosphocholine (di14:1 PC); 1,2-Dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine (di16:1 PE); 1,2-Dipentadecanoyl-*sn*-glycero-3-phosphoglycerol (sodium salt) (di15:0 PG); 1,2-Dimyristoyl-*sn*-glycero-3-phospho-L-serine (sodium salt) (di14:0 PS); 1,2-Dimyristoyl-*sn*-glycero-3-phosphate (sodium salt) (di14:0 PA); 1,1′,2,2′-Tetramyristoyl cardiolipin (T14:0 CL); N-Lauroyl sphingomyelin (N12:0 SM); N-Heptadecanoyl ceramide (N17:0 Cer); 1-Heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (17:0 lysoPC); 1,2,3,4-$^{13}C_4$-Palmitoyl-L-carnitine hydrochloride ($^{13}C_4$-16:0 CN) (Sigma-Aldrich, St. Louis, MO).

2.2. Preparation of platelets and microparticles

Washed platelets were prepared from 3 patients with ovarian cancer and 3 gender and age-matched healthy subjects. Blood samples from cancer patient were obtained prior to any surgical intervention or chemotherapy. Patients with ovarian cancer did not have any detectable metastasis at the time of blood draw, and had normal blood counts and normal routine coagulation parameters. Platelets were prepared from 20 mL of fresh whole blood using a previously described method [21]. PMPs were prepared by activating the washed platelets with a combination of thrombin (0.1 unit/mL) and collagen (50 μg/mL) at 37 °C for 30 min, as described before [3,18]. Washed platelets and PMPs were pelleted and stored at −80 °C for lipidomics.

2.3. Shotgun lipidomics

Both platelets and PMPs were resuspended in 300 μL PBS and homogenized for 1 min using a disposable soft tissue homogenizer. An aliquot of 25 μL was pipetted to determine the protein content (BCA protein assay kit, Thermo Scientific, Rockford, IL). The rest of homogenate was transferred into a disposable glass culture test tube. For quantification of all reported lipid species, a mixture of lipid internal standards was added prior to lipid extraction. Lipid extraction was performed by a modified Bligh and Dyer procedure [29]. Each lipid extract was resuspended into a volume of 500 μL of chloroform/methanol (1:1, *v*/*v*) per mg of protein and flushed with nitrogen, capped, and stored at −20 °C for lipid analysis. For direct infusion electrospray ionization (ESI) analysis, lipid extract was further diluted to a final concentration of ~500 fmol/μL, and the mass spectrometric analysis was performed on a QqQ mass spectrometer (Thermo TSQ VANTAGE, San Jose, CA) equipped with an automated nanospray device (TriVersa NanoMate, Advion Bioscience Ltd., Ithaca, NY).

2.4. Western blot analysis

Washed platelets were lyzed in a buffer consisting of 1% Triton X-100, 50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM Na pyrophosphate, 1 mM Na_3VO_4, 10% glycerol, and freshly added protease and phosphatase inhibitors from Roche Applied Science (Cat. # 05056489001 and 04906837001, respectively). Protein concentrations of the lysates were determined by a BCA Protein Reagent Kit (Pierce Biotech.), and 25 μg of proteins were subjected to gel electrophoresis on 10% SDS-PAGE gels. Antibodies used were against LPP1 (Abcam, ab198280, at 1:250 dilution), and β-ACTIN (Sigma-Aldrich, A5316, 1:5000).

2.5. Statistical analysis

All lipid class or species data were expressed as means ± SE for triplicate measurements. Comparisons between groups were made using the Student's *t*-test with $p < 0.1$ being considered statistically significant. For comparable analysis between platelets and PMPs, the measured value for a lipid class or a species was converted to parts per thousand (ppt), or equivalent to nmol per a total of 1000 nmol lipids. Measured values for lipid classes in platelet and PMP samples were subjected to multivariate analysis in the form of unsupervised principal component analysis (PCA), using Partek Genomics Suite 6.6 (Partek, St. Louis, MO). PCA is used to visualize the interrelationship between large numbers of measured (observed) variables and describes the largest variation in data using principal components to plot against each other so that trends and groupings can be detected [10].

3. Results

3.1. Lipid content of platelets/PMPs in ovarian cancer patients and normal subjects

A total of 12 classes and 177 species of lipids were analyzed by shotgun lipidomics. The total amount of lipid content of platelets and PMPs was not different between cancer patients and normal subjects (Fig. S1A). The total amount of lipid content in PMPs was less than in platelets, which is expected given the size difference between platelets and PMPs.

The 2 main lipid classes in both platelets and PMPs were phosphatidylethanolamine (PE), including plasmalogen PE (PE-pl), and phosphatidylcholine (PC) (Fig. 1), similar to the reported lipid composition of cell membrane in eukaryotes [26].

Lipid profiles (classes and species) of platelets and PMPs were compared between cancer and control groups. Phosphatidylinositol (PI) class in PMPs was significantly higher (~2-fold) in cancer patients than in controls, and a similar trend was observed in platelets although statistically non-significant ($p = 0.20$). Lyso-phosphatidylcholine (LPC) class was ~26% lower in cancer platelets compared to controls ($p = 0.09$), and a similar trend was observed in PMPs ($p = 0.18$) (Fig. 1). In cancer platelets, a trend toward reduction in PC, a precursor of LPC, was observed.

Changes in lipid species in platelets or PMPs of ovarian cancer patients as compared to healthy subjects were compiled in Table 1. To conduct a meaningful comparison of lipid species, we focused on lipid species in 7 lipid classes with a known regulatory role in coagulation (PS, PI, PA, PG, PC, CAR, and SM), and eliminated the other 5 lipid classes (ceramides, cardiolipins, PE, lyso-PE, Lyso-PC) from our analysis. In the