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Endothelial, platelet, and tissue factor-bearing microparticles in cancer patients with and without venous thromboembolism

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ABSTRACT

Background: Cancer is a prothrombotic state, with an increased prevalence of venous thromboembolism (VTE). Microparticles (MPs) are sub-micron-sized vesicles derived from activated or apoptotic cells that may play a role in VTE, although evidence of this association is still limited.
Objectives: To evaluate the hypothesis that elevated numbers of endothelial (EMPs), platelets (PMPs), and Tissue Factor-bearing MPs (TF^+MPs) in plasma may contribute to cancer-associated thrombosis.
Patients/Methods: EMPs, PMPs and TF^+MPs plasma levels were measured in 90 consecutive patients (cases) referred to our Department (30 with a first episode of unprovoked VTE; 30 with active cancer; 30 with a diagnosis of acute VTE associated with active cancer), and in a group of 90 healthy subjects (controls). MPs analyses were performed by flow-cytometry (Cytomics FC500).
Results: Cases showed statistically significant higher (mean ± SD) circulating EMPs and PMPs plasma levels (920 ± 341 and 1221 ± 413 MP/μL, respectively) than controls (299 ± 102 and 495 ± 241 MP/μL; $p<0.005$). Moreover cancer patients (with and without VTE) showed higher (mean ± SD) TF^+MPs (927 ± 415 MPs/μL) than controls (204 ± 112 MPs/μL; $p<0.001$). The subgroup of cancer patients plus VTE showed statistically significant higher TF^+MPs plasma levels (1019 ± 656 MPs/μL) than cancer patients without VTE (755 ± 391 MPs/μL, $p=0.002$). Multivariate analysis failed to show a significant association between elevated TF^+MPs and VTE in cancer patients.
Conclusions: Our results suggest that MPs might be an important intermediate in the cascade of cellular injury and vascular dysfunctions underlying the process of thrombosis, particularly in cancer. Further clinical investigations are needed to confirm the precise role of MPs in predicting hypercoagulable state in patients with cancer.

Introduction

Microparticles (MPs) are phospholipid vesicles, smaller than 1 μm in diameter, derived mainly from blood and endothelial cells, in response to activation or apoptosis. MPs release is an integral part of the membrane-remodelling process in which the asymmetric distribution of constitutive phospholipids between the two leaflets is lost [1]. MPs are detected and characterized on the basis of antigens characteristic of their respective parental cells [2]. Found at the electronic microscopy since 1967 by Wolf and coll. [3], MPs were considered "cellular dust" without any biological function. Recently it has been hypothesized that MPs play a role in inflammation, coagulation and vascular function [4], and it was demonstrated that these elements could induce cell signalling [5] and regulate many pathophysiological processes [6] including neoangiogenesis [7]. Moreover, a procoagulant function has been attributed to MPs, due to the presence phospholipids on their outer surface, in particular phosphatidilserine (PS), which could induce the activation of the coagulation cascade. This procoagulant role is amplified by the capability of PS to activate tissue factor (TF) [8]. Elevated MPs of different phenotypes have been documented in the blood of patients with venous thromboembolism (VTE) [9–11] and with various diseases characterized by arterial and venous thrombotic complications (i.e. heparin induced thrombocytopenia [12], cardiovascular diseases [13,14], thrombotic thrombocytopenic purpura [15], sickle cells diseases [16], uraemia [17], diabetes [18] and anti-phospholipid antibody syndrome [19]). Thromboembolic disease is a well recognized complication of cancer. Clinical studies reported that approximately 5–15% of all cancer patients develop thrombotic events

Abbreviations: MPs, microparticles; EMPs, endothelial microparticles; PMPs, platelets microparticles; TF^+, MPs tissue factor-bearing microparticles; VTE, venous thromboembolism; TF, tissue factor; PS, phosphatidilserine; SS, side scatter; FS, forward scatter.

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[20,21]. However, despite this strong association between VTE and malignant diseases, the molecular and cellular bases of this relationship remain uncertain. Recent studies have shown that cancer patients with VTE have higher MPs levels than cancer patients without thrombosis [22–25]. In this case-control study we evaluated the role of endothelial (E-), platelet (P-) and TF^+MPs in the development of cancer-associated thrombosis.

Material and Methods

Out of 145 patients consecutively admitted from January 2007 to June 2008 to the Thrombotic Unit and Oncology Department at the Padua University Hospital, 90 patients (M/F 42/48; age range 40-92 yrs) were enrolled in the study: thirty patients (M/F 12/18; age range 43-79 yrs) with a first episode of objectively confirmed acute unprovoked VTE [at diagnosis, and before anticoagulant therapy was started]; 30 (M/F 15/15; age range 45-89 yrs) with active cancer [diagnosis less than six months before blood sample collection or under chemotherapy]; 30 (M/F 15/15, age range 40-92 yrs) admitted with a diagnosis of acute VTE associated with active cancer.

Subjects under 18 years of age (4), without informed consent (8), with inactive or non documented cancer (12), with expectancy of life below 3 months (4), and with conditions known to be characterized by elevated MPs plasma levels (27) [i.e. sepsis, acute infection, pregnancy, acute coronary syndrome, heparin-induced thrombocytopenia, antiphospholipid antibody syndrome, thrombotic thrombocytopenic purpura, transient ischemic attack, severe hypertension, diabetes, recent abdominal and thoracic major surgery or multiple sclerosis] were excluded.

Ninety healthy subjects, partners or friends of cases, who never experienced thrombosis and without history of cancer, sex (M/F 42/48) and age (range 35-95 yrs) matched to cases, served as controls.

After clean puncture of an antecubital vein with a 21-gauge needle, 18 mL of venous blood were drawn without applying venostasis into a syringe pre-filled with 2 mL of sodium citrate 109 mol/L.

Platelet-free plasma (PFP) samples were obtained by two sequential centrifugations (15 min at 1500×g each one) at room temperature, frozen in 1.5 mL tubes as 500 μL aliquots, and stored at -80 °C until use [26]. Samples were processed within 1 to 3 hours from collection and analyzed immediately after thawing.

For EMPs labelling, 10 μL of CD146-phycoerythrin-cyanin 5.1 (PC5) (Beckman Coulter, Miami, Florida) and 10 μL of annexin V-fluorescein isothiocyanate (FITC) prediluted 1:2 in binding buffer (Bender MedSystems GmbH, Vienna, Austria) were added to 30 μL of freshly thawed PFP. For PMPs labelling 10 μL of CD61-phycoerythrin (PE) (Beckman Coulter, Miami, Florida) and 10 μL of annexin V-FITC prediluted 1:2 in binding buffer were added to 30 μL of freshly thawed PFP. For TF^+MPs 10 μL of mAb anti-TF-PE (BD, Biosciences, Milan, Italy) and 10 μL of annexin V-FITC prediluted 1:2 in binding buffer were added to 30 μL of freshly thawed PFP. After 30 min of incubation at room temperature, samples were diluted in 500 μL of Annexin-V kit binding buffer. The isotype controls used were IgG1-PC5, clone MOPC-21 (BioLegend Europe, The Netherlands), IgG1-PE, clone MOPC-21 (BD Biosciences); mouse IgG1-FITC, clone MOPC-21 (BioLegend Europe). Standardization of MPs count was achieved using a blend of monodisperse fluorescent beads of three diameters (0.5, 0.9 and 3 μm) (Megamix, BioCytex, Stago, France). Each individual bead subset was gated on the basis of their side scatter (SS) and FL1 properties; with discriminator on forward scatter (FS), the MPs region was then set up in the lower part using the gate of 0.5 μm bead and in the upper part by the 0.9 μm one.

Thirty microliters of counting beads with an established concentration (Flow Count™ Fluorospheres, Beckman Coulter, Miami, Florida) were added to each sample in order to calculate MPs as absolute numbers per microliter of PFP. MPs analyses were performed on Cytomics FC500 flow cytometer (Beckman Coulter, Miami Florida) as previous described [27].

The measurement of MPs procoagulant activity in plasma was performed using a functional assay, the Zymuphen MP-Activity ELISA kit (Hyphen Biomed, Neuville-sur-oise, France) following the manufacturer instructions. Briefly, procoagulant MPs were captured by immobilized Annexin V, and the anionic phospholipid content was determined by a prothrombinase assay; results are expressed as nanomolar PS equivalent (nM PS) with reference to a standard curve constructed with liposomes of known PS concentrations.

Statistical analysis

Statistical analysis was performed using commercially available statistics software (Statistica Vers. 5.0; Stat Soft, Tulsa, OK, USA). The sample size calculation was based on our laboratory previous observations and the following assumption: *i*) Expected SD of EMPs in control group 100 MPs/μL; ii) power = 95%; iii) alpha = 0.01. Based on these assumptions we needed a control group of at least 25 patients. Mean value and standard deviation (SD) of quantitative variables were calculated and differences between cases and controls were evaluated using the non-parametric Mann-Whitney U test. Spearman's correlation test was used to determine correlation coefficients. To estimate the strength of the association between elevated levels of TF^+MPs and VTE unadjusted Odds Ratio (OR) and 95% confidence intervals (CI) were computed. Logistic regression was performed to assess the relationship between MPs and VTE after adjusting for potential confounders like sex, age, chemotherapy, tumor site and platelet count. All tests for statistical significance were two-tailed and *p* values less than 0.05 were considered statistically significant.

Results

MPs in patients and controls

Enrolment of study patients is reported in Fig. 1. The total study population, consisted of 90 patients, showed higher (mean ± SD) circulating EMPs and PMPs plasma levels (920 ± 341 and 1221 ± 413 MPs/μL, respectively) than controls (299 ± 102 and 495 ± 241 MPs/μL). The difference was statistically significant ($p = 0.005$ and 0.001, respectively). The analysis of sex-specific differences showed a statistically significant higher PMPs level (mean ± SD) in female (1324 ± 712 MPs/μL) than in male (1089 ± 821 MPs/μL; $p = 0.037$) patients. Accordingly, females showed higher mean platelet plasma levels (278 ± 51 $x10^9$/L) than males (251 ± 47 $x10^9$/L); the difference was not statistically significant ($p = 0.68$). No statistically significant difference was found in mean EMPs plasma levels between males and females. Moreover, no statistically significant correlation was found between EMPs or PMPs and age, neither in cases nor in controls (data not shown).

MPs in patients with cancer

Cancer patients with and without VTE (n. 60 subjects) showed higher (mean ± SD) EMPs (1010 ± 639 MPs/μL), PMPs (968 ± 482 MPs/μL), and TF^+MPs (927 ± 415 MPs/μL) than controls (299 ± 102 MPs/μL, 495 ± 241 MPs/μL, and 204 ± 112 MPs/μL, respectively) [Fig. 2]. The differences were statistically significant in all the three comparisons considered ($p < 0.001$). The subgroup of cancer patients plus VTE showed statistically significant higher TF^+MPs plasma levels (1019 ± 656 MPs/μL) than cancer patients without VTE (755 ± 391 MPs/μL, $p = 0.002$) [Fig. 3]. Considering the group of patients with cancer, no sex-specific differences or significant linear correlation between MPs plasma levels and age were found (data not shown). On the contrary, a statistically significant correlation was evidenced