CELULAR ORIGIN OF CIRCULATING MICROPARTICLES IN PATIENTS WITH VENOUS THROMBOEMBOLISM AND CANCER

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Abstract:

Microparticles (MPs) are extracellular vesicles considered to be powerful cellular effectors. They are present in healthy individuals and are elevated in pathological conditions such as inflammatory and neoplastic diseases, and thrombosis. The relationship between venous thromboembolism (VTE) and cancer has been well established. MPs are thought to be a pathogenic connection between the two entities. If confirmed, they could be used as biomarkers.

Our aim was to characterize the MPs in both diseases according to their cellular origin (cellular, endothelial, platelet, leukocyte and those that exhibited mucin 1 on their surface). Functional parameters such as D-dimer (DD) and soluble P-selectin (sPsel) were also studied.

96 patients with idiopathic VTE and 85 with advanced lung, stomach or pancreatic neoplasia were considered. All of them were followed clinically for two years and those who were diagnosed with cancer in the VTE group or those who developed thrombosis in the group of neoplastic patients were excluded from the study. Finally, 82 VTE patients and 68 cancer patients were analyzed.

In our results, we found that total MPs and platelet-derived MPs differentiated both patient groups. Additionally, significantly greater numbers of DD and sPsel ($p < 0.001$) were determined in the VTE group.

The differences found between both groups, taking into account the origin of the MPs, could be caused by the prothrombotic characteristics of the neoplastic group and their sequestration within active clots in the VTE group.

Key words: microparticles, cancer, deep vein thrombosis, pulmonary embolism, coagulation.

ORIGEN CELULAR DE MICROPARTÍCULAS CIRCULANTES EN PACIENTES CON ENFERMEDAD TROMBOEMBÓLICA VENOSA Y CÁNCER

Resumen

Las micropartículas (MPs) son unas vesículas extracelulares consideradas potentes efectores celulares. Están presentes en individuos sanos y se encuentran elevadas en estados patológicos como enfermedades inflamatorias, neoplásicas y trombosis. La relación entre enfermedad tromboembólica venosa (ETV) y cáncer está bien establecida. Se piensa que las MPs serían una conexión patogénica entre ambas entidades. De confirmarse, podrían utilizarse como biomarcadores.

Nuestro objetivo fue caracterizar las MPs en ambas patologías atendiendo a su origen celular (celular, endotelial, plaquetar, leucocitario y las que exhibían en su superficie mucina 1). También se estudiaron parámetros funcionales como el dímero D (DD) y la P-selectina soluble (sPS).

Se consideraron 96 pacientes con ETV idiopática y 85 con neoplasias avanzadas de pulmón, gástrico o páncreas. A todos ellos se les realizó un seguimiento clínico de dos años en el que se excluyeron del estudio aquellos que fueron diagnosticados de cáncer en el grupo de ETV o que desarrollaron trombosis en el grupo de pacientes neoplásicos. Finalmente, se analizaron 82 pacientes con ETV y 68 con cáncer.

En nuestros resultados encontramos que las MPs totales y las MPs de origen plaquetar diferenciaban ambos grupos de pacientes. Además, se determinaron cifras significativamente mayores de DD y sPS ($p < 0{,}001$) en el grupo de ETV.

Las diferencias encontradas entre ambos grupos, teniendo en cuenta el origen de las MPs, podrían estar causadas por las características protrombóticas del grupo neoplásico y por el secuestro de las mismas dentro de los coágulos activos en el grupo de ETV.

Palabras clave: Micropartículas, cáncer, trombosis venosa profunda, embolia pulmonar, coagulación.

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INTRODUCTION

Microparticles (MPs) are microvesicles measuring between 0.1 and 1 μm, released by cells during activation, damage or apoptosis[1-5]. Initially, they were considered to be devoid of any biological function, but now they are considered to be involved in various pathophysiological processes, such as hemostatic processes, inflammation or cell migration[6-9]. MPs are present in healthy individuals, but are found in high levels in patients with different inflammatory, neoplastic and vascular diseases, including arterial and venous thrombosis[9-13]. Experimental studies with animal models have shown that certain types of MPs can accelerate thrombus formation in vivo[14]. High levels of MPs have been found in neoplastic patients [15] and it has been postulated that these could largely explain the association between the occurrence of thrombotic phenomena and cancer. This association has been firmly established and there has been data to support it for many decades now[1, 2]. Venous thromboembolism (VTE) is involved in comorbidity and mortality in cancer patients. In fact, pulmonary embolism (PE) is the second leading cause of death among neoplastic patients, only behind neoplasia itself[16]. Not all neoplasia has the same risk of thrombosis. Advanced stage adenocarcinomas of pancreatic, gastric or pulmonary origin are high risk profiles.[5]. In addition, up to 15% of idiopathic VTE precede a cancer diagnosis, which could cause thrombosis. The search for biomarkers for the early diagnosis of neoplasia in thrombosis patients and/or that can predict the risk of VTE in cancer patients is of great clinical interest. D-dimer (DD) is a fibrin degradation product and therefore a direct marker of fibrinolysis whose values are elevated in thrombotic processes in which fibrinolysis is activated. DD is included among the diagnostic algorithms for VTE in the general population, but its role as a biomarker of thrombosis in cancer patients has not been demonstrated. MPs are other possible biomarkers that have garnered the interest of researchers and clinicians, although they have yet to be implemented.

We believe that characterizing MPs in both these diseases—VTE and cancer—could provide useful information for future designs of prospective studies which could look into the predictive role of MPs in terms of diagnosis of thrombotic complications in cancer patients and diagnosis of hidden cancer in idiopathic VTE. We consider that an initial approximation could be evaluating MPs according to the cellular origin of these diseases of disparate etiopathogenesis but associated in medical practice.

Our objective was to compare the characterization of MPs according to their cellular origin in idiopathic VTE patients and non-VTE-associated cancer patients, aside from determining other functional parameters.

MATERIALS AND METHODS

Design: prospective observational study of two patient groups; one made up of idiopathic VTE patients and another made up of patients with advanced lung, stomach or pancreatic cancer not associated with VTE. Circulating MP levels and their cellular origin were quantified in both populations.

Patients:

Group of VTE patients

Inclusion criteria: ambulatory patients diagnosed with deep vein thrombosis (DVT) or patients diagnosed with PE who were not diagnosed with cancer during the two-year follow-up. Patients who started anticoagulant therapy up to 72 h prior to inclusion in the study were admitted.
Exclusion criteria: patients diagnosed with neoplasia during the follow-up period. The search protocol for hidden neoplasia in VTE patients was the one according to Jara-Palomares et al[17].

Group of cancer patients

Inclusion criteria: patients with a cytological and histological diagnosis of Stage IV or IIIB non-small cell lung carcinoma, with pleural or pericardial effusion, or with stomach or metastatic pancreatic carcinoma, from outpatient oncology consultations. These patients had not had any previous VTE-related events and were not on treatment or prophylaxis with low-molecular-weight-heparins.

Exclusion criteria: cancer patients diagnosed by means of objective testing for VTE (DVT and/or PE) or arterial thrombosis during the follow-up period.

All patients considered for the study signed informed consent forms.

Blood sampling: all patients had their blood drawn using a 21-gauge needle, without using compression or with minimal venous occlusion, in plastic blood collection tubes with 0.109 M of buffered trisodium citrate solution (Vacutainer ®, BD Biosciences, Erembodegem, Belgium) discarding the first 3 mL. The samples were processed within the first hour after extraction; they were centrifuged at 1,500 g for 30 min at 4°C with no brake to obtain plasma containing MPs and platelet-free plasma (PFP). The PFP was divided into aliquots and stored at -80 ºC until their analysis.

Quantification and characterization of MPs by flow cytometry: the levels of total MPs and platelet-derived MPs in plasma were determined in all patients using the LSRFortessa flow cytometer (BD Biosciences, Erembodegem, Belgium). For detection of total MPs, annexin V conjugated with CF Blue (Immunostep, Salamanca, Spain), was used. It recognizes and binds to phosphatidylserine present on the microparticle surface. For detection of platelet-derived MPs (PDMPs) and endothelial cell-derived microparticles (EDMPs), the human antibodies anti-CD31 and anti-CD41 conjugated with fluorescein isothiocyanate (FITC) and phycoerythrin cyanine 7 (PECy7), respectively (Beckman Coulter, Marseille, France), were used. For detection of leukocyte-derived MPs (LDMPs), the human antibody anti-CD45 FITC (Beckman Coulter, Marseille, France) was used. For detection of MPs with mucin 1 expression on the surface (MPs-MUC1+), the human antibody anti-CD227 FITC (BD Biosciences, Erembodegem, Belgium) was used.

Flow cytometer calibration for MP detection was done using the mix of Megamix-Plus SSC fluorescent beads (Biocytex, Marseille, France), according to the manufacturer's specifications. The MPs were defined as events collected within the established region for MPs and positive for annexin V. The PDMPs were identified as positive MPs for the anti-CD31 and anti-CD41 antibodies; the EDMPs as positive MPs for CD31 and negative for CD41; the LDMPs as positive MPs for CD45; and the MPs-MUC1+ as positive MPs for CD227. As a negative control, the isotype controls corresponding to each antibody were used. Counting beads (6 μm) with a concentration of around 1,000 beads/μL (Perfect-Count Microspheres; Cytognos, Salamanca, Spain) were used to calculate the concentration of MPs from the absolute counts of cells. Results were expressed as events/mL.

Determination of D-dimer and soluble P-selectin: D-dimer (DD) was determined using the immunoturbidimetric method STA-Liatest D-Di (Diagnostic Stago, Parsippany, New York, USA), using an STA-R analyzer. Results were expressed in mg/mL.

The levels of soluble P-selectin (sPsel) in plasma were detected using ELISA (Human sP-selectin/CD62P Immunoassay; RD Systems, Minneapolis, MN, USA). Results were expressed in ng/mL.

Statistical analysis: quantitative results were expressed as mean and standard deviation while qualitative results were expressed as absolute values and frequencies. The paired Student's T-test was used for the comparison of quantitative variables between different clinical groups, while the Chi-squared test was used for the comparison of qualitative variables. A significance level of $p < 0.05$ was used. The results were analyzed using the SPSS 22 statistical software package for Windows.

RESULTS

From January 2011 to November 2013, 181 patients were recruited. There were 96 in the group of VTE patients, of which 14 were finally excluded owing to a diagnosis of neoplasia during the two-year follow-up, and there were 85 patients in the neoplastic group, of which 17 were excluded due to the development of a thrombotic process during the follow-up period. Therefore, 82 patients with idiopathic VTE and 68 patients diagnosed with cancer were analyzed. In the latter group, 56 (82.4%) died during the follow-up period. None of them had an episode of VTE during follow-up and thrombosis was not the cause of death.

The cancer patients were younger ($p = 0.026$), predominantly male ($p = 0.018$), smokers ($p <0.001$) and more frequently had a drinking habit (34%). At the time of recruitment, 45 (66 %) were undergoing different chemotherapy regimens. The idiopathic VTE patients were older, with hypertension ($p = 0.001$), with 45% being women. Table 1 summarizes the clinical characteristics of both populations.

With regard to the MP populations studied, total MP levels that were significantly higher were found in the neoplastic patients in comparison to the patients diagnosed with VTE (cancer: $26{,}454 \times 10^3 \pm 4{,}238 \times 10^3$; VTE: $14{,}787 \times 10^3 \pm 1{,}146 \times 10^3$; $p = 0.01$). A similar pattern was observed in the

number of PDMPs (cancer: 22,263x10^3 ± 3,973x10^3; VTE: 11,118x10^3 ± 1,004x10^3; p = 0.008). No differences were seen for the rest of the cellular origins studied (endothelial and leukocyte) and for the MPs-MUC-1+ (Table 2).

With regard to the levels of DD and sPsel, both parameters were found to be significantly higher in the group of VTE patients in comparison to the cancer patients (p <0.001; Table 2).

Table 1. Clinical characteristics of patients with idiopathic venous thromboembolism (VTE) and cancer. BMI: body mass index; SD: standard deviation.

Characteristics	Idiopathic VTE (n = 82)	Cancer (n = 68)
Age, years (mean ± SD)	63.4 ± 14.5	62.3 ± 11.3
Masculine gender n (%)	45 (54.9)	50 (73.5)
BMI (kg/m2) (mean ± SD)	31.0 ± (5.4)	26.6 ± 5.6
Smoker, n (%)	27 (32.9)	52 (76.5)
Alcohol consumption, n (%)	10 (12.2)	23 (33.8)
Hypertension, n (%)	44 (53.7)	17 (25.0)
Dyslipidemia, n (%)	22 (26.8)	23 (33.8)
Diabetes, n (%)	13 (15.9)	9 (13.2)
Chronic respiratory disease, n (%)	20 (24.4)	15 (22.1)
Ischemic cardiomyopathy , n (%)	7 (8.5)	2 (2.9)
Chronic renal insufficiency, n (%)	5 (6.1)	6 (8.8)
Dementia, n (%)	13 (16.0)	6 (8.8)

Table 2. Circulating microparticles (MPs) of cellular origin and other functional parameters in patients with venous thromboembolism (VTE) and cancer. EDMPs: Endothelial-derived MPs; PMPs: Platelet-derived MPs; LDMPs: Leukocyte-derived MPs; MPs-MUC1+: MPs that exhibited mucin 1 on their surface; sP-selectin: Soluble P-selectin. The results were expressed as the mean ± standard deviation. Significance level: p < 0.05.

Parameters	VTE (n = 82)	Cancer (n = 68)	P value
Total MPs/mL (x103)	14787 ± 1146	26454 ± 4238	0.01
EDMPs/mL	27027 ± 3170	25432 ± 2056	0.687
PDMPs/mL (x103)	11118 ± 1004	22263 ± 3973	0.008
LDMPs/mL	98705 ± 9104	96818 ± 9147	0.885
MPs-MUC1+/mL	135118 ± 41377	251606 ± 91310	0.239
D-dimer (µg/L)	6869 ± 944	1327 ± 227	< 0.001
sP-selectin (ng/mL)	58.92 ± 3.21	40.80 ± (2.06)	< 0.001

DISCUSSION

In the present study, we compared the levels of different MP populations between two patient groups—one made up of patients diagnosed with idiopathic VTE and another one made up of non-thrombosis-associated cancer patients. Additionally, DD and sPsel levels were also compared. In our results, we observed total MP and PDMP levels that were surprisingly much higher in the group of cancer patients than in the VTE group, and DD and sPsel levels that were much higher in the thrombosis group than in the cancer group. The PDMPs are considered to be procoagulant microparticles that are involved in the initiation and propagation of venous thromboembolism[18, 19]. PDMPs are known to be elevated in VTE patients[20]. Nevertheless, several authors point out that finding elevated levels in VTE depends on the group with which the comparison is made[21]. These authors compared PDMP levels in a group of patients diagnosed with PE with healthy subjects, with and without cardiovascular risk factors. They only found significant differences between PE patients and healthy subjects with no cardiovascular risk factors. In the group with PE, blood was drawn right before starting anticoagulant treatment.

This does not coincide with our study, in which blood was drawn up to 72 hours after the start of anticoagulant treatment. In our context, it would not have been viable to use a study design in which blood was drawn before the start of anticoagulant treatment, as clinical practice guidelines allow for the start of treatment prior to the confirmation of diagnosis[22]. In addition, the patients comprising our cancer group were those who had the highest number of procoagulant MPs in other published series[23]. Therefore, the difference found in our study should not be considered in absolute terms. Several aspects must be taken into account, such as comparison with a group of cancer patients with high procoagulant risk, the time of blood sampling, which may be within 72 hours of anticoagulant treatment of the VTE patient, or that a large part of circulating PDMPs in patients with an active thrombotic process may be trapped in the clots, leading to a false decrease in circulating PMs.

Although the MPs-MUC1+ did not refer to any specific cell lineage, they were determined in order to identify the MPs derived from tumor cells, particularly adenocarcinoma. It has been described that tumor cells can overexpress at the MUC1 membrane level, so MPs with this origin could be identified using this type of antigen. Nevertheless, we found no differences between the VTE group and the cancer group in our results. Other authors found higher levels of MPs-MUC1+ in cancer patients[24]. This discrepancy could be due to the patient profile chosen for each study, the type of neoplasia or the stage, which could affect MUC1 expression in tumor cells. In addition, although tumor cells can overexpress MUC1 on the surface, this protein is not exclusive to tumor cells. Other types of cells can give rise to MPs-MUC1+, as this protein is also involved in the immune system, trapping pathogens[25].

DD is one of the markers used in diagnostic algorithms for VTE, both in their DVT and PE expression. Clinical trials and prospective studies[26] with thousands of patients support the high negative predictive value of DD. However, the diagnostic value of DD in cancer patients is called into question. Although DD is highly sensitive for thrombosis, it is not specific at all, as it can also be elevated in pro-inflammatory and neoplastic situations. Particularly, doubt has been cast as to whether the cut-off level of 500 mg/L is suitable for a population with cancer. In our group of patients with cancer without VTE, mean DD was 1.327 ± 227 mg/L. This may suggest that a cut-off point above 1,000 mg/L is perhaps more suitable for this group.

sPsel is a protein involved in cell adhesion functions, stored in intracytoplasmic granules of platelets and endothelial cells. When these cells are activated, Psel is translocated to the plasma membrane, enabling interaction with its ligands[27]. It plays a fundamental role in the initial migration of leukocytes to the focus of inflammation, but it is also important in platelet aggregation through platelet-fibrin and platelet-platelet bridges to the area of vascular damage[28]. Thrombin is one of the stimuli for Psel to be secreted by the endothelial cells. But the role of Psel in tumor metastatic processes, facilitating the process of migration and dissemination of tumor cells, has also been recognized[29]. In our study groups, the determination of sPsel has a behavior similar to that of DD, which indicates that, although it could be elevated in patients with metastatic cancer, it is also significantly elevated in acute VTE. All our patients were in the advanced stages of their neoplasia, although we do not know the specific cancer treatment they were undergoing and whether this could affect the sPsel levels. The potential role of this protein in VTE diagnosis has been suggested[30]. Other authors have pointed out its predictive role in VTE diagnosis in cancer patients[31]. Adding biomarkers such as DD and sPsel to the Khorana prediction scale improved diagnostic precision. The authors advocate two cut-off points for both biomarkers with potential value in predicting thrombotic events in cancer patients; DD ³1,440 mg/L and sPsel ³53.1 ng/mL. These figures are consistent with the means we obtained in our sample of cancer patients without VTE, which were below them.

Based on our results, we can say that certain populations of circulating MPs such as total MP levels and PDMP levels could differentiate between patients with idiopathic VTE and non-VTE-associated cancer patients. These observations would need to be validated in future cohort studies with cancer patients and VTE patients focused on the role of MPs as biomarkers of thrombotic events in cancer patients and hidden neoplasia in VTE patients. When interpreting our results, it is important to bear in mind that it is a preliminary study with a small sample size, in which VTE patients who were already undergoing anticoagulant treatment—although for less than 72 h—at the time of blood sampling, were admitted.

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