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Prevention of microvesiculation by adhesion of buds to the mother cell membrane — A possible anticoagulant effect of healthy donor plasma ☆

Mojca Frank [a], Mateja Manček-Keber [b], Mojca Kržan [c], Snežna Sodin-Šemrl [a], Roman Jerala [b], Aleš Iglič [d], Blaž Rozman [a], Veronika Kralj-Iglič [e,*]

[a] Department of Rheumatology, University Medical Centre Ljubljana, Ljubljana, Slovenia
[b] Department of Biotechnology, National Institute of Chemistry, Ljubljana, Slovenia
[c] Institute of Pharmacology and Experimental Toxicology, Faculty of Medicine, University of Ljubljana, Slovenia
[d] Laboratory of Physics, Faculty of Electrical Engineering, University of Ljubljana, Slovenia
[e] Laboratory of Clinical Biophysics, Faculty of Medicine, University of Ljubljana, Slovenia

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Abstract

Microvesicles (MVs) found in peripheral blood are derived from the budding of cell membranes and are associated with a higher risk of thrombosis. Recently, a hypothesis has been suggested that certain plasma proteins could suppress microvesiculation by mediating adhesion of the buds to the mother cell membrane. In a pilot study, we have tested this hypothesis by considering the relation between the amount of MVs in peripheral blood and the ability of plasma to induce adhesion between giant phospholipid vesicles (GPVs). MVs were isolated from human plasma and counted by flow cytometry. The adhesion between GPVs was measured by assessing the average angle of contact between the adhered vesicles. It was found that greater ability of plasma to induce adhesion relates to smaller concentration of MVs in plasma. The ratio between the concentration of MVs and the concentration of platelets proved the most efficient parameter to predict the propensity of the membrane to shed vesicles. Our results indicate that a stronger attractive interaction between GPVs mediated by plasma is associated with a smaller amount of MVs per platelets. Plasma that mediates stronger attractive interaction between GPVs might potentially be associated with a smaller risk of thrombosis.

Keywords: Microvesicles; Microparticles; Coagulation; Thrombosis

Contents

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* Corresponding author: Laboratory of Clinical Biophysics, Faculty of Medicine, University of Ljubljana, Slovenia. Tel.: +386 41 720 766; fax: +386 1 476 88 50.
E-mail address: veronika.kralj-iglic@fe.uni-lj.si (V. Kralj-Iglič).

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1. Introduction

Microvesicles (MVs) are derived from cell membranes as final products of the budding process [1] induced by cell activation [2], shear stress in the circulation [3] and apoptosis [4]. They were found to have important roles in vascular haemostasis [5], promotion of cancer [6] and inflammation [7]. Increased levels of circulating MVs were found in patients suffering from cardiovascular disorders [8], cancer [9], infection [10] and autoimmune diseases [11], among them antiphospholipid syndrome (APS) [12]. The prothrombotic role of MVs was indicated by the presence of thromboembolic events in these diseases.

The abundance of MVs in peripheral blood can be quantitatively assessed which defines them as a potential diagnostic tool. However, for this purpose, the understanding of the underlying mechanisms should be improved. The abundance of MVs reflects the extent of the membrane pool available for budding and vesiculation, the properties of the membrane, that render it more likely to bud and vesiculate, and the properties of the surrounding solution. The dissolved compounds not only enter into chemical reactions with membrane bound receptors, but also may affect the budding and vesiculation process by physical mechanisms [13]. The membrane budding and vesiculation in the presence of different compounds can be studied in a simple *in vitro* system of giant phospholipid vesicles (GPVs), as vesicle shape changes can be observed live under phase contrast microscope. In observing the effect of plasma protein $\beta_2$glycoprotein I (β_2GPI) on the budding GPVs, it was found that β_2GPI-mediated attractive interaction between the bud and mother vesicle may cause adhesion of the bud to the mother membrane, thereby preventing the bud to become an exovesicle [13]. As a similar process may take place also in cells, a possible anticoagulant mechanism was suggested [13]. According to the hypothesis, the compounds that mediate a short-ranged attractive interaction between membranes are anticoagulant, while the compounds that impair this mechanism are procoagulant.

Here, we tested, in a pilot study, the hypothesis that human plasma-mediated attractive interaction between membranes represents a mechanism preventing the release of MVs into the circulation. We suggest a method by which the mediating effect of human plasma is quantitatively assessed by the average angle of contact between adhered GPVs, due to the addition of plasma, while the ability of membrane to release MVs is assessed by the ratio f=MV concentration/platelet concentration.

2. Materials and methods

2.1. Microvesicles and platelet concentration

After 12 hour fasting, 1.5 ml of venous blood was drawn from two authors (assigned A and B) and a volunteer (C), who gave a written consent, into vacutubes with 0.129 M trisodium citrate. Following the centrifugation of blood (1500 g, 15 min, 20 °C, SIGMA 3 K15 Centrifuge, Sigma) 500 μl of human plasma was removed for isolation of MV according to the method described in Diamant et al. [14] and 10 μl of plasma was used for the experiments with GPVs. The platelet concentration in blood samples anticoagulated with K_3EDTA was determined by Beckmann Coulter HMX, Fullerton, California, USA.

2.2. Flow cytometry

Samples containing MVs were analysed using a Coulter EPICS Altra flow cytometer (Beckman Coulter Electronics). 7000 events were recorded. Plasma MV concentration was calculated using Flow-count fluorospheres (FS) (1.05×10^6/ml, Beckman Coulter) for calibration

according to the equation: plasma MV concentration (MVs/ml) = total number of MVs counted / total number of FS counted × FS assayed concentration × dilution. FS assayed concentration was 5.25×10^4/ml. Two samples from each subject were analysed and a difference in MV concentration smaller than 3% was obtained.

2.3. Preparation and observation of giant phospholipid vesicles

GPVs were prepared by the modified electroformation method (originally proposed by Angelova et al. [15]), as described in [16]. For GPVs containing 10% weight ratio of cardiolipin, synthetic lipids 1, 1′ 2, 2′-tetraoleoyl cardiolipin, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and cholesterol (Avanti Polar Lipids, Inc.), all dissolved in a 2:1 chloroform/methanol mixture, were combined in the proportion of 1:7:2. The GPVs were rinsed out of the electroformation chamber with the solution containing 9:1 glucose (192 mM)/PBS (277 mM) mixture. The GPVs were observed by an inverted microscope Zeiss Axiovert 200 with phase contrast optics and recorded by the Sony XC77CE video camera. For experiments 5 μl of citrated plasma was added to the solution of GPVs (45 μl). The experiments were performed at 37 °C. The images were observed at 6, 10, 15, 20 and 25 min after the addition of plasma.

2.4. Determination of the mediating effect of plasma

To assess the strength of the adhesion between GPVs after the addition of plasma all clearly visible angles of contact (in degrees) between adhered GPVs [17] from an image were measured, using Image J software. Stronger adhesion is consistent with larger angles of contact and it yields a larger area of contact between the two vesicles. The angle Y was calculated as the average of all clearly visible contact angles measured from all the micrographs taken within 1 min. Adhesion angles from different subjects were compared using Student t test with $p < 0.05$ being taken as statistically significant.

3. Results

3.1. The effect of plasma on GPVs

The addition of human plasma to the suspension of GPVs induced the adhesion of GPVs (Fig. 1a) in a timescale of minutes. Fig. 1a shows how the angles of contact between adhered GPVs were measured.

3.2. Time course of the adhesion angle between GPVs

The adhesion angles of subjects A and B, Y_A and Y_B respectively were compared at 6, 10, 15, 20 and 25 min after the addition of plasma to the GPVs obtained from the same sample. At all defined times Y_B was found to be significantly larger than Y_A ($3 \times 10^{-9} < p < 0.044$). We considered the method sensitive enough to distinguish between subjects.

3.3. Comparison between adhesion angle and plasma MV concentration

Plasma MV concentration in the three subjects was related to the adhesion angle Y (Fig. 1b). It can be seen roughly that higher MV concentration indicates a weaker adhesion between membranes (smaller Y) as subject A in which the MV concentration was the highest had the smallest Y. In subjects B and C, however, larger Y did not correspond to lower MV concentration (Fig. 1b). These results indicated that more data on the system should be included to better explain the ability of membrane pool to release MVs reflected in the adhesion angle Y. Therefore we considered also the size of the vesiculating membrane pool. As it was found that in normal subjects about 80% of MVs originate from platelets [14] we represented the size of the vesiculating membrane pool by the platelet concentration. The three subjects differed considerably in the platelet concentration, which were 214×10^9/l,

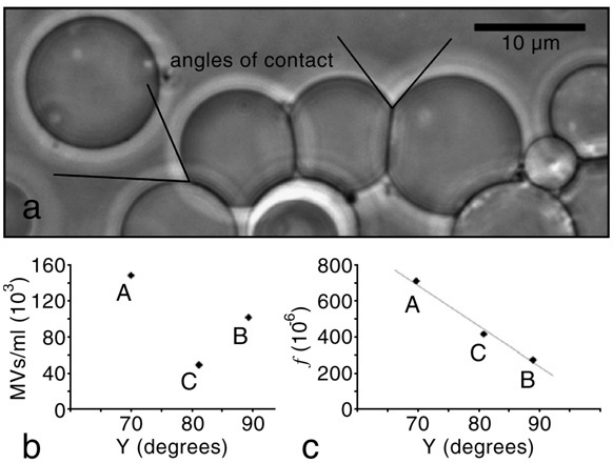

Fig. 1. a. Adhesion of GPVs after the addition of plasma to the suspension of GPVs with indicated angles of contact between GPVs. b. The dependence of plasma MV concentration on the adhesion angle (Y) measured 25 min after the addition of plasma from subjects A, B and C. c. The dependence of the ratio f = MV concentration/platelet concentration, indicating the ability of the membrane pool to shed MVs, on the adhesion angle (Y) measured 25 min after the addition of plasma from subjects A, B and C.

394×10^9/l and 120×10^9/l in subjects A, B and C, respectively.

To assess the ability of the membrane to shed vesicles we used the ratio = MV concentration/platelet concentration (parameter *f*). Fig. 1c shows an excellent correspondence between the ability of the membrane to shed vesicles (*f*) and the adhesion angle *Y*. A negative relationship can be observed: *f* is smaller if *Y* is larger (Fig. 1c). It was therefore concluded that the parameter *f* and not the plasma MV concentration could be efficient for reflecting the ability of membrane pool to shed MVs.

4. Discussion

Circulating MV concentration is influenced by (1) the size of the pool of cells that are able to vesiculate, (2) the intrinsic properties of cell membranes and (3) the mediating effect of plasma or plasma proteins on the adhesion between membranes. In the present work the amount of shed MVs from the three subjects with no record of previous or present thromboembolic events, cancer, infection and autoimmune diseases was compared and the differences were explained by considering two of the three above mentioned factors: the platelet concentration representing the size of the vesiculating pool and the mediating effect of plasma on the adhesion between membranes. The ratio *f* as a measure of the ability of platelet membrane to vesiculate was found to be smaller, if the mediating effect of plasma, represented by adhesion angle (*Y*) was larger. The results presented are in favor of the hypothesis that plasma-mediated attractive interaction between membranes could represent an anticoagulant mechanism by preventing the release of MVs into the circulation. A larger number of subjects are planned to be enrolled in the study in the future. At the moment the methodology for the determination of the mediating effect of plasma is inadequate for the analysis of a larger number of subjects due to the small amounts of GPVs produced as described above. The method of GPVs production is being improved also in order to yield samples containing GPVs with more uniform and repeatable sizes and densities.

Platelets represent the major pool of vesiculating cell membranes in healthy subjects [14] as well as in many pathologic disorders [reviewed in 5,18,19]. Because MVs are derived from the budding of platelet membranes, in healthy subjects, lower platelet concentration would imply lower plasma MV concentration and relatively uniform values of *f* within the population. In contrast, an increased concentration of platelet-derived MVs and low concentration of platelets were found in different disorders, such as heparin-induced thrombocytopenia (HIT) [18, reviewed in 20] and idiopathic thrombocytopenic purpura [19]. Furthermore, a low concentration of red blood cells and a high concentration of red blood cell-derived MVs were found in haemolytic anaemias, such as thalassemia [21].

In some of these disorders, such as idiopathic thrombocytopenic purpura [22] and thalassemia [21], a strong budding process leading to cell lysis occurs. In these cases, high MV concentration would be associated with a low concentration of vesiculating cells. High values of the parameter *f* could therefore be a convenient indicator of enhanced cell destruction in cytopenic states due to its enhanced sensitivity: the numerator (MV concentration) is increased and the denominator (vesiculating cell concentration) is decreased, both contributing to the increase of *f*.

The parameter *f* could be an indicator of enhanced platelet activation with shedding of MVs in thrombocytopenic states associated with hypercoagulability and thrombosis. It is known that HIT patients with severe thrombocytopenia are at increased risk of thrombosis [23] and increased concentration of potentially procoagulant platelet-derived MVs was reported in acute HIT patients [18]. Also, the presence of antiphospholipid antibodies (aPL) was correlated to the increased risk of thrombosis in aPL-associated thrombocytopenia patients [24]. As aPL were shown to be involved in platelet activation, which could be accompanied by enhanced platelet microvesiculation [25], platelet-derived MVs could be potentially increased in aPL-associated thrombocytopenia. In contrast, thrombocytopenia was not found to be related to the increased risk of thrombosis in APS patients [26]. It was suggested that circulating MVs isolated from APS patients are mainly endothelium-derived [12].

In conclusion, plasma and/or plasma protein-mediated attractive interaction between membranes has been proposed as a possible anticoagulant mechanism, preventing the release of procoagulant MVs into the circulation. This mechanism might be impaired in APS and other prothrombotic states with elevated concentration of circulating MVs. The ratio between the concentration of cell-derived MVs and the concentration of cells that preferentially vesiculate was used as a measure of the ability of cell membranes to vesiculate and should be further studied in evaluating the role of MVs in disease pathogenesis. It could be an indicator of enhanced cell destruction, as well as of thrombotic propensity

in different disorders. For additional readings on this concept of the Mosaic of Autoimmunity, we refer to several recently published papers [27–40].

Take-home messages

- This pilot study indicates that larger ratio between plasma microvesicle concentration and platelet concentration is consistent with the smaller ability of plasma to mediate the adhesion between membranes.
- Plasma-mediated adhesion between membranes of the bud and mother cell suppresses the shedding of microvesicles from the mother cell membrane and in this respect represents an anticoagulant mechanism.
- The mediating effect of different samples such as human plasma on the adhesion between membranes can be determined *in vitro* by assessing an average angle of contact between giant phospholipid vesicles that adhered after sample addition.
- In prothrombotic states with elevated concentration of circulating MVs this protective mechanism might be impaired.

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Low-dose peptide tolerance therapy of lupus generates plasmacytoid dendritic cells that cause expansion of autoantigen-specific regulatory T cells and contraction of inflammatory Th17 cells

Subnanomolar doses of an unaltered, naturally occurring nucleosomal histone peptide epitope, H4(71-94), when injected s.c. into lupus-prone mice, markedly prolong lifespan by generating CD4+25+ and CD8+ regulatory T cells (Treg) producing TGF-β. Here, Kang HK. et al. (J Immunol 2007; 178: 7849-58) demonstrated that the induced Treg cells suppress nuclear autoantigen-specific Th and B cells and block renal inflammation. Splenic dendritic cells (DC) captured the s.c.-injected H4 (71-94) peptide rapidly and expressed a tolerogenic phenotype. The DC of the tolerized animal, especially plasmacytoid DC, produced increased amounts of TGF- β, but diminished IL-6 on stimulation via the TLR-9 pathway by nucleosome autoantigen and other ligands; and those plasmacytoid DC blocked lupus autoimmune disease by simultaneously inducing autoantigen-specific Treg and suppressing inflammatory Th17 cells that infiltrated the kidneys of untreated lupus mice. Low-dose tolerance with H4 (71-94) was effective even though the lupus immune system is spontaneously preprimed to react to the autoepitope. Thus, H4(71-94) peptide tolerance therapy that preferentially targets pathogenic autoimmune cells could spare lupus patients from chronically receiving toxic agents or global immuno-suppressants and maintain remission by restoring autoantigen-specific Treg cells.