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# Suppression of membrane microvesiculation — A possible anticoagulant and anti-tumor progression effect of heparin

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ABSTRACT

Heparins (unfractionated and low molecular weight (LMWH) heparins) primarily used as anticoagulants, were found to be effective also in slowing down the development of some types of cancer. On the other hand, the number of microvesicles in the peripheral blood originating from the budding of cell membranes (mostly platelets) is increased in hypercoagulabile states as well as in cancer, indicating a possible common underlying mechanism. It was hypothesized that by mediating an attractive interaction between phospholipid membranes heparin suppresses microvesiculation and thereby acts as an anticoagulant and anti-tumor agent. In this work, the effect of LMWH nadroparin on phospholipid membranes was tested *in vitro* in a system of giant phospholipid vesicles (GPVs) created by electroformation and observed under the phase contrast microscope. Plasma of different blood donors containing different concentrations of nadroparin was added to the suspension of GPVs to induce adhesion between GPVs. The attractive interaction between membranes was assessed by measuring the average effective angle of contact between the adhered GPVs. It was found in healthy donors, in a donor with gastrointestinal cancer and in a donor with rheumatoid arthritis that adding therapeutic doses of nadroparin to the plasma samples enhanced adhesion of phospholipid membranes in a dose and time-dependent manner while nadroparin alone had no effect within the therapeutic concentration range. The results are in favor of the hypothesis that suppression of microvesiculation underlies both, the anticoagulant and the anti-tumor progression effect of heparin.



## Introduction

Thrombosis often occurs as a consequence of cancer. If occult, the state was in a strict sense defined as the Trousseau syndrome [1]. The term now includes all hypercoagulabile states associated with cancer [2,3]. Heparins (unfractionated and LMWHs) being the effective choice for prevention and treatment of thromboembolism [4], revealed also a beneficial effect by suppressing the tumor progression in some types of cancer [5–10]. Other anticoagulants were considered, however, in many cases they did not prove as effective as heparin [5,6].

To explain the effect of heparin in the treatment of the Trousseau syndrome, several mechanisms were suggested, mainly pointing to its role in biochemical reactions involved in the blood clot development and interaction of free cancer cells with distal endothelial cells [11]. Understanding of the complex role of heparin in various physiological processes related to the Trousseau syndrome remains yet rudimentary.

In this work we point to a non-specific biophysical mechanism which could be involved in both, coagulation and tumor progression, i.e. to microvesiculation of phospholipid membranes. Microvesicles (microparticles) that are pinched off the membranes of different cells [12–16] and travel with circulation, can be viewed upon as extracellular organelles which may convey matter and information to distal cells. By exposing tissue factor and anionic phospholipids on the surface for blood clot production, they act as catalytic surfaces for reactions involved in coagulation mechanisms [17–19]. It was found that the number of microvesicles in peripheral blood is increased in different disorders such as thromboembolism, autoimmune diseases (e.g. antiphospholipid syndrome) [20], cancer [21] and type II diabetes mellitus [22].

To better understand the underlying mechanisms, membranes of giant phospholipid vesicles (GPVs) are studied since they mimic some

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essential features of cell membranes while their dimensions and properties enable observation live under the optical microscope. Vesiculation of the GPV membrane can be induced by controlling the environmental conditions, so it is possible to distinguish between specific effects caused by exogenously added substances.

Recently, a study on the effect of plasma phospholipid-binding protein β2-glycoprotein I (a cofactor for antiphospholipid antibodies in antiphospholipid syndrome) and antiphospholipid antibodies on vesiculation of GPVs showed that budding and vesiculation of the membrane can be affected by the solution in which the GPVs are immersed [23]. Namely, molecules and ions in the solution may mediate attractive interaction between membranes and cause adhesion, the effect depending on the surface charge of the membrane. This was observed experimentally and described theoretically [24,25]. The adhesion was quantitized by introducing the effective angle of contact between the adhered vesicles [26]. Measuring all clearly visible effective angles of contact in an image and calculating their average value give a measure for the attractive interaction between the membranes ($Y$), where larger angle $Y$ exhibits larger mediating effect [26,27]. The mediating effect was revealed in β2-glycoprotein I which was previously found to be an anticoagulant [28], while antiphospholipid antibodies (which are considered procoagulant) diminished the effect of β2-glycoprotein I [23]. Further study showed that in the budding process, the bud can be (due to the mediated attractive interaction between the membrane of the bud and of the mother vesicle), adhering to the mother membrane if the mediating effect of the solution is strong enough [24]. It was suggested that similar mechanisms may take place in cells and that substances, which mediate attractive interaction between membranes and prevent pinching off the buds from the mother membrane, are suppressors of microvesiculation. A hypothesis was put forward on the anticoagulant effect of plasma proteins [24]. As microvesicles were suggested to play a role also in tumor progression, the above hypothesis was redefined to include both the anticoagulant and the anti-tumor progression effect of plasma proteins. As microvesicles are promoters of blood coagulation and tumor progression, any substance that suppresses microvesiculation would act as an anticoagulant and anti-tumor-progression agent [29].

On the basis of clinical evidence indicating anticoagulant and anti-tumor progression effect of heparin and on the basis of the hypothesis on mediated attractive interaction between membranes, we suggest that the mediating effect of heparin would represent a possible explanation for the observed clinical features.

In this work we investigated the effect of LMWH nadroparin on the adhesion of GPVs. The effect of nadroparin dissolved in sugar solution and of nadroparin dissolved in plasma samples of different blood donors was induced by adding the sample to the suspension of GPVs in the sugar solution and allowing the GPVs to adhere to each other. The adhesion was assessed by the average effective angle of contact between GPVs. We hypothesized that nadroparin would induce adhesion between GPVs in concentrations that are comparable to therapeutic concentrations in peripheral blood, the effect being more pronounced (the average effective angle of contact being larger) for higher concentrations of nadroparin.

### *Patients*

Six (6) donors who gave a written informed consent were included in the study. One patient (female, 36 years of age) was diagnosed with gastrointestinal cancer at the Department of Gastroenterology, University Medical Centre Ljubljana, the other patient (female, 54 years) was diagnosed with rheumatoid arthritis at the Department of Rheumatology, University Medical Centre Ljubljana while four persons were healthy individuals; two males (57 and 30 years, respectively) and two females (62 and 65 years, respectively).

## Materials and methods

### *Chemicals*

LMWH nadroparin calcium (Fraxiparine Forte, 19.000 UI AXa/ml) was from GlaxoSmithKline UK, while synthetic phospholipids (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,1_2,2_-tetraoleoyl cardiolipin) and plant cholesterol were from Avanti Polar Lipids, Inc.

### *Isolation of plasma*

1.6 ml of venous blood was collected into vacutubes (BD Vacutainers) containing 0.129 M trisodium citrate (drawn blood volume to citrate volume ratio = 4:1) and processed within 15 min. Following the centrifugation of blood (1550×*g*, 20 min, 20 °C, SIGMA 3K18 Centrifuge, Sigma), 300 μl of plasma was removed to be used for experiments with GPVs. Plasma was frozen at −80 °C until experiments with GPVs were performed.

### *Preparation of GPVs*

GPVs were prepared by the modified electroformation method, originally proposed by Angelova et al. [30]: POPC and cholesterol, both dissolved in a chloroform/methanol mixture at a concentration of 1 mg/ml, were combined in the proportion of 4:1 (v/v). 20 μl of lipid mixture was applied to each of the two platinum electrodes shaped as rods (approximate length 4 cm and diameter 1 mm). The electrodes were left in a low vacuum for 2 h for solvent to evaporate. The lipid-coated electrodes were thereafter placed into a microcentrifuge tube filled with 2 ml of 0.3 M sucrose solution to form an electroformation chamber. AC electric current with an amplitude of 5 V and a frequency of 10 Hz was applied to the electrodes for 2 h, which was followed by 2.5 V and 5 Hz for 15 min, 2.5 V and 2.5 Hz for 15 min and finally 1 V and 1 Hz for 15 min. After the electroformation, 600 μl of sucrose solution containing GPVs and 1 ml of 0.3 M glucose solution were pipetted into each of the three 2 ml plastic microcentrifuge tubes, which were afterwards sealed with parafilm band to prevent the entrance of air and to protect the solution from microorganisms. Vesicles were left for sedimentation and stabilisation under gravity at room temperature for 1 day.

### *Preparation of samples*

4 μl of citrated plasma, 4 μl of 0.3 M sugar solution (glucose:sucrose ratio was 5:3) containing nadroparin and 32 μl of GPV suspension were added in the plastic tube in the above consecutive order. Immediately after the addition of GPVs, the solution was transferred through a circular opening into CoverWell™ Perfusion Chamber (Grace Bio-Labs). The experiments were performed at room temperature. The final dilutions of nadroparin (19.000 UI AXa/ml) used in the experiments with GPVs were 1:80 (237.5 UI AXa/ml), 1:500 (38 UI AXa/ml), 1:5000 (3.8 UI AXa/ml) or 1:7000 (2.7 UI AXa/ml). The dilution 1:7000 is within the therapeutical range of subcutaneous nadroparin concentrations used for treatment of thromboembolic disorders.

### *Determination of the mediating effect of samples*

The solution containing GPVs was observed by the inverted microscope Nikon Eclipse T-300 with phase contrast optics. For all experiments, the images of adhered GPVs were taken at different times, at least 4 min after mixing GPVs with plasma samples and heparin. GPVs adhered to each other in the timescale of minutes after the addition of the sample composed of plasma and heparin, the effect

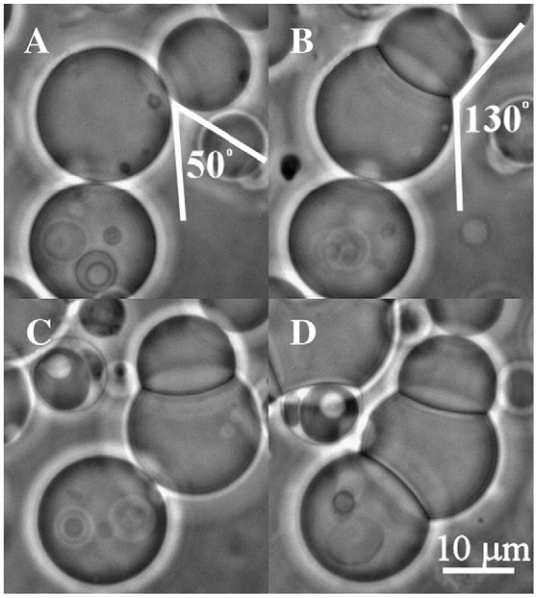


**Fig. 1.** Coalescence (adhesion) of GPVs after the addition of the sample containing nadroparin and human plasma. The time interval between subsequent pictures is 2 min. The assessment of the effective angle of contact ($Y$) is indicated. It can be seen that $Y$ increases with time.

enhancing with time (Fig. 1 and 2). Several images of GPVs were acquired along the middle line of the round perfusion chamber in the timespan of 1 min. The process of acquisition was repeated two or three times. To assess the adhesion between GPVs after the addition of plasma (+nadroparin), all clearly visible effective angles of contact between adhered GPVs were measured from the representative images using the ImageJ software.

*Statistical analysis*

The mean value and the standard deviation of the measured effective angles of contact ($Y$) were calculated by using the Microsoft Excel software. The values of the average effective angle of contact within each GPV sample at different times after the addition of plasma (+nadroparin) were compared by using the $t$-test.

## Results

Introduction of plasma into the suspension containing GPVs caused adhesion between GPVs. The average effective angle of contact between GPVs ($Y$) reached the value $67°\pm21°$ in 20 min (assessed from $n=371$ clearly visible contacts) and increased further in the next 20 min up to $74°\pm25°$ ($n=644$). The effect was stronger when therapeutic concentration of nadroparin (dilution 1:7000) and plasma were added to the suspension of GPVs, where the respective values of $Y$ were $76°\pm20°$ after 20 min ($n=354$) and $102°\pm21°$ after 40 min ($n=589$), respectively. Higher concentration of nadroparin in plasma (dilution 1:500) yielded for $Y$ $83°\pm21°$ ($n=464$) after 20 min and $106°\pm20°$ ($n=637$) after 40 min. All the differences between the average effective angles of contact, assessed at the same conditions but at different times were statistically significant ($p<0.001$).

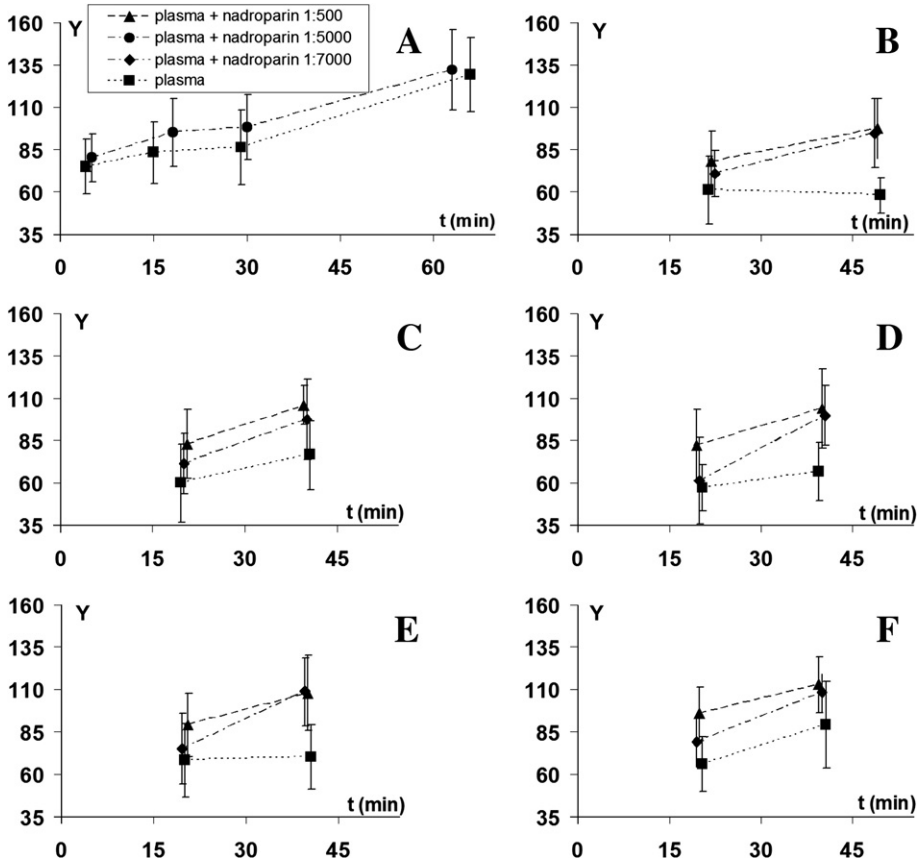


**Fig. 2.** Time course of the average effective angle of contact between adhered vesicles ($Y$) after the addition of the sample to GPVs. The final dilution of the added nadroparin is indicated in the figure. Plasma of different blood donors was used in experiments: a patient with gastrointestinal cancer (A), a patient with rheumatoid arthritis (B), healthy donors (C–F).

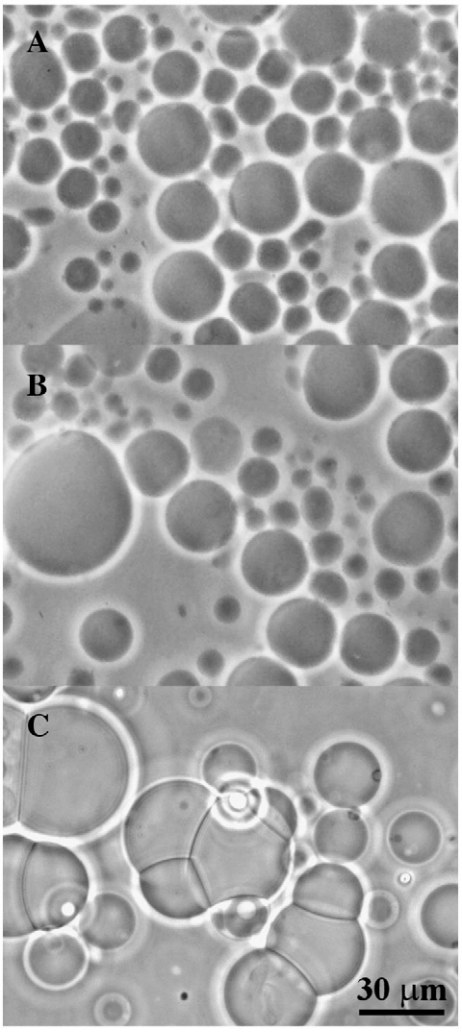


**Fig. 3.** GPVs 70 min after addition of therapeutical concentration of nadroparin (1:7000 (2.7 UI AXa/ml)) (A) and 75 min after addition of 14× therapeutical concentration of nadroparin (1:500 (38 UI AXa/ml)) (B) showing no adhesion between GPVs. At dilution 1:80 (238 UI AXa/ml), adhesion between GPVs took place around 20 min after addition of nadroparin (C).

Nadroparin alone, dissolved in sugar solution at dilutions of 1:7000 and 1:500, failed to induce adhesion of vesicles (Figs. 3A, B). The mediating effect of nadroparin was however revealed at significantly higher concentration (dilution 1:80).

## Discussion

Our results indicate that the addition of heparin to the plasma increases the ability of plasma constitutients to mediate attractive interaction between membranes. Heparin in the sugar solution exhibits the same effect, albeit too weak to have the impact in the therapeutic concentrations. The effect was found in all plasma samples considered: in healthy subjects and in both patients (with rheumatoid arthritis and with gastrointestinal cancer). It was recently also shown in the system of GPVs, β2-glycoprotein I and anti-β2-glycoprotein I IgG antibodies (isolated from a serum of an antiphospholipid syndrome patient with the history of thrombosis), that β2-glycoprotein I-mediated membrane adhesion was significantly reduced in the presence of anti-β2-glycoprotein I IgG antibodies while therapeutic concentration of nadroparin completely restored (rescued) the adhesion [31]. All these results are in favor of the hypothesis of the nonspecific anticoagulant and anti-tumor-progression effect of plasma constituents through suppression of membrane vesiculation.

To explain why heparin in plasma significantly affects adhesion while the same concentrations in sugar solution have no effect, it was interpreted that plasma already contains molecules that mediate attractive interaction between membranes, while added heparin enhances the effect.

In order to explain the intriguing anti-tumor-progression effect of heparin, various mechanisms were suggested. An acknowledged mechanism of metastatic potential is based on a scenario where after intravasation, a metastatic cancer cell passes into bloodstream where it forms a microembolus composed of the metastatic cell, platelets and leukocytes. The microembolus travels to the vessel of the target organ where the cell interacts with the endothelium of the blood vessel and passes to the organ, where it can form metastasis. It was suggested that heparin affects the interaction of a free malignant cell with platelets rendering it more accessible to leukocytes and diminishing its probability to adhere to endothelium [6,32]. The underlying mechanism was suggested to be based on blocking of selectins by heparin.

Another mechanism was proposed to be based on fibrinolytic activity of heparin. It was suggested [33] that fibrin mesh may serve as a support for metastatic cells which is in line with the results of experiments, indicating that fibrinolytic agents are effective agents for reduction of metastases [32,34].

Furthermore, it was reported [35] that heparin uptake into cancer cells can be promoted by conjugation to poly (β-amino ester)s. Detailed studies [36] suggest that poly (β-amino ester)–heparin complexes affect cellular processes including the induction of transcription factor and caspase activation. Internalized heparin is considered cytotoxic, causing cancer cell death by inducing apoptosis [36,37].

However, none of the hitherto proposed mechanisms explains both, the anticoagulant and the anti-tumor-progression effects. In contrast, both effects can be explained by suppression of the microvesiculation which can according to our hypothesis be induced by mediating the attractive interaction between membranes of the bud and of the mother cell. Here we present evidence that heparin enhances the attractive interaction and can according to the hypothesis [24] act as a suppressor of microvesiculation. Furthermore, there are indications that heparin is also an anti-inflammatory agent [38] and was proven efficient also in treatment of acute thrombosis and obstetric complications in antiphospholipid syndrome [31], which comprises yet other mechanisms that are associated with microvesiculation.

The direct evidence of the suppression of budding was obtained with artificial membranes having a controlled composition of lipids [24]. In cells, the outer membrane layer is coated by glycans which render the system intrinsically distinct. It remains to be elucidated which mechanisms are essential in the budding of cell membranes, and to which extent the phospholipid bilayer reflects the features in the membranes. It should be pointed out that the attractive interaction between membranes mediated by the molecules in the solution, is of a short range (or the order of nanometers or even smaller) and therefore applies to structures which are already very close together (such as the bud and the mother membrane). This was confirmed by theoretical models of the mediated attractive interaction between membranes [39,25].

## Conclusions

Our results indicate that mediating attractive interaction between membranes is a possible mechanism of microvesiculation suppression of heparin, which may underlie its anticoagulant, anti-tumor-progression and anti-inflammatory effect.

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