

# Effects of Low-Molecular-Weight Heparin on Adhesion and Vesiculation of Phospholipid Membranes

## A Possible Mechanism for the Treatment of Hypercoagulability in Antiphospholipid Syndrome

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Heparins represent an efficient treatment of acute thrombosis and obstetric complications in antiphospholipid syndrome (APS). Enhanced microvesiculation of cell membranes, as detected by reduced membrane adhesion, can contribute to hypercoagulability in APS. Healthy donor IgG antibodies significantly increased  $\beta$ 2-glycoprotein I ( $\beta$ 2-GPI)-induced membrane adhesion, indicating that IgG antibodies might supplement the role of  $\beta$ 2-GPI in the regulation of membrane microvesiculation in healthy individuals. Anti- $\beta$ 2-GPI IgG antibodies significantly reduced  $\beta$ 2-GPI-induced membrane adhesion, suggesting a direct role of anti- $\beta$ 2-GPI antibodies in enhancing membrane microvesiculation in APS. Therapeutic concentration of nadroparin completely restored  $\beta$ 2-GPI-induced membrane adhesion in the presence of anti- $\beta$ 2-GPI IgG antibodies. A novel anticoagulant mechanism of nadroparin in APS is suggested that supplements its direct effect on the coagulation cascade. Restoration of adhesion between negatively charged membranes in the presence of nadroparin might decrease shedding of microvesicles into the surrounding solution and could thus contribute to the efficacy of heparin treatment in APS.

**Key words:** low-molecular-weight heparin; anti- $\beta$ 2-glycoprotein I antibodies; phospholipid membranes; adhesion; budding; microvesicles; microparticles

### Introduction

Antiphospholipid syndrome (APS) is characterized by arterial and venous thromboses, pregnancy morbidity, and thrombocytopenia in the presence of antiphospholipid antibodies (aPL). The obstetric complications (recurrent pregnancy loss, intrauterine growth restriction,

and preeclampsia) seen in APS are a consequence of thrombosis in placental and decidual circulation as well as of the direct effect of aPL on trophoblast cells.<sup>1</sup> Treatment of thrombosis in APS involves anticoagulation, initially with intravenous heparin or low-molecular-weight heparin (LMWH), followed by lifelong therapy with oral warfarin. Heparin (either unfractionated or LMWH) in combination with aspirin is widely accepted as the treatment of choice in pregnancies complicated with APS. A live-birth rate of 75% is expected when aPL-positive women with recurrent pregnancy loss

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are treated with standard heparin plus aspirin, and similar live-birth rates could be achieved with LMWHs.<sup>2,3</sup> The beneficial effects of heparins in APS cannot be merely explained by their direct effect on the coagulation cascade. Several other mechanisms were found to contribute to the efficacy of heparin in the treatment of APS.<sup>4-9</sup>

Microvesiculation of cell membranes is yet an underappreciated and poorly understood mechanism. Enhanced microvesiculation of cell membranes was implicated as one of the mechanisms responsible for the hypercoagulability seen in APS<sup>10</sup> as well as other prothrombotic disorders, including thrombotic thrombocytopenic purpura, heparin-induced thrombocytopenia, and myocardial infarction (as reviewed in Ref. 10). Microvesicles (microparticles) are derived from an exocytic budding process of cell membranes. They are procoagulant as a result of the surface exposure of anionic phospholipids and tissue factor that provide a catalytic surface for coagulation reactions.<sup>11</sup> Increased levels of circulating endothelial<sup>10,12-14</sup> as well as platelet-derived<sup>12</sup> microvesicles were isolated from plasma of aPL-positive patients who experienced thrombotic complications. An elevated number of endothelial microvesicles was found in a proportion of women with a history of pregnancy loss, although aPL-positive women were excluded from the study.<sup>15</sup> Preeclampsia was associated with an increased number of circulating syncytiotrophoblast microvesicles that are believed to promote a systemic inflammatory response and maternal endothelium dysfunction, characteristic of the disorder.<sup>16</sup> Treatment of pregnant mice with phosphatidylserine-containing phospholipid vesicles induced placental vessel thrombosis and led to intrauterine growth restriction.<sup>17</sup>

Microvesicle production is a hallmark of cell activation. Several authors reported that aPL isolated from APS patients induce platelet and endothelial cell activation, resulting in proadhesive and procoagulant cell phenotypes (as reviewed in Ref. 18). Cultured endothelial cells

were shown to specifically release microvesicles with high procoagulant activity when treated with plasma of APS patients.<sup>10</sup> In an *in vitro* model of a single giant phospholipid vesicle (GPV), anti- $\beta$ 2-glycoprotein I (anti- $\beta$ 2-GPI) antibodies from an APS patient in the presence of  $\beta$ 2-GPI directly enhanced budding of the negatively charged GPV membrane and induced membrane vesiculation.<sup>19</sup>

Recently, we had proposed that a nonspecific mechanism based on mediated attractive interactions between phospholipid membranes could play an important role in the microvesiculation process, consequently affecting hemostasis.<sup>20</sup> According to the hypothesis, attractive interactions between phospholipid membranes in the presence of certain plasma proteins, especially  $\beta$ 2-GPI, might lead to the adhesion of buds to the mother-cell membrane. This would prevent the detachment of buds from the cell membrane surface and their release into the surrounding solution, directly decreasing the extent of membrane microvesiculation.<sup>20</sup> The hypothesis was studied theoretically and experimentally. The attractive interactions between like-charged membranes were explained within the theory of two interacting, electric, double layers as a consequence of an orientational ordering of mediating polyions in the gradient of the electric field.<sup>21-23</sup> Orientational ordering of particles (polyions) with extended charges yields a bridging configuration of a polyion that is energetically most favorable. In the equilibrium the free energy attains its minimum corresponding to an equilibrium distance between the membranes. The equilibrium distance is of nanometer order, appearing as adhesion between lipid bilayers, and the interaction is of an electrostatic origin. Direct experimental evidence supporting the hypothesis was found in the *in vitro* model of a budding GPV in which  $\beta$ 2-GPI was shown to mediate strong attractive interaction between phospholipid membranes and to induce the adhesion of the temperature-induced bud to the negatively charged mother vesicle.<sup>24</sup> Because a similar process might be involved in budding

and vesiculation of cell membranes *in vivo*, it was speculated that human plasma samples (in the presence of which membrane adhesion was stronger)<sup>25</sup> would contain a smaller number of microvesicles. A statistically significant negative correlation was found between the number of microvesicles per platelet (isolated from plasma samples of healthy donors<sup>26</sup> as well as from gastrointestinal patients)<sup>27</sup> and the strength of adhesion between phospholipid membranes after the addition of the plasma samples.

$\beta$ 2-GPI, the major antigen for aPL in APS is strongly implicated in adhesion of phospholipid membranes and was therefore considered to be an anticoagulant. Binding of anti- $\beta$ 2-GPI antibodies to membrane-bound  $\beta$ 2-GPI might importantly interfere with  $\beta$ 2-GPI-induced adhesion between membranes, resulting in an increased microvesiculation in APS. In the present work the interactions between the phospholipid membrane,  $\beta$ 2-GPI, healthy donor IgG antibodies, IgG fraction from an APS patient, purified anti- $\beta$ 2-GPI IgG antibodies from an APS patient, and LMWH nadroparin were investigated in the GPV model. Specifically, the relevance of these interactions to processes of membrane adhesion and vesiculation was addressed to better understand the role of aPL in enhanced membrane vesiculation and the efficacy of LMWHs in the treatment of APS.

## Materials and Methods

### Preparation of GPVs

The lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS), and plant cholesterol (Avanti Polar Lipids Inc., Alabaster, AL, USA) were dissolved in chloroform at a concentration of 1 mg/mL. For neutral GPVs, POPC and cholesterol were combined in the proportion of 4:1 (v/v), and for negatively charged phosphatidylserine-containing GPVs (POPS-GPVs) POPC, cholesterol, and POPS were combined in the proportion of 7:2:1 (v/v/v). GPVs were

prepared by the modified electroformation method (originally proposed by Angelova *et al.*<sup>28</sup>), as described in Janša *et al.*<sup>27</sup> After the electroformation, 600  $\mu$ L of 0.2 mol/L sucrose solution containing electroformed GPVs was added to 1 mL of 0.2 mol/L glucose solution, and the vesicles were left to sediment under gravity in a low vacuum at room temperature for 1 day. Before the experiments, the sucrose/glucose suspension containing GPVs was modified by the addition of 0.28 mol/L PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 1.5 mmol/L  $\text{KH}_2\text{PO}_4$ , 7.8 mmol/L  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , pH = 7.4). The final suspension of GPVs used in the experiments, therefore, contained 0.14 mol/L glucose, 0.14 mol/L sucrose, and 0.084 mol/L PBS.

### $\beta$ 2-GPI, IgG Antibodies, and Nadroparin

$\beta$ 2-GPI was isolated from pooled human plasma by a slightly modified method, as described previously,<sup>29</sup> and was concentrated using Microcon centrifugal filter device with YM-30 Ultracel membrane (30,000 nominal molecular weight limit) (Millipore, Billerica, MA, USA). Aliquots of  $\beta$ 2-GPI in PBS (5.9 mg/mL) were stored at  $-20^\circ\text{C}$ . The final concentration of  $\beta$ 2-GPI in the experiments was 55  $\mu\text{g/mL}$ , which is approximately half of free  $\beta$ 2-GPI concentration in human plasma. In the experiments where the influence of  $\beta$ 2-GPI concentration on membrane adhesion was tested, the final concentrations of  $\beta$ 2-GPI ranged from 4.6–250  $\mu\text{g/mL}$ . IgG fractions from sera of a healthy donor and a syphilitic patient were isolated by affinity purification on a 2-mL protein G column (ImmunoPure(G) IgG purification kit; Pierce Chemical, Rockford, IL, USA), using the protocol recommended by the manufacturer, and were equilibrated against PBS, pH 7.4, in a desalting column. The syphilitic IgG fraction contained only high titers of anticardiolipin antibodies (aCL). The final concentration of purified healthy donor IgG antibodies in the experiments ranged from

10.6–77  $\mu\text{g/mL}$ , and the final concentration of the syphilitic IgG fraction was 77  $\mu\text{g/mL}$ . The IgG fraction of a patient with primary APS was obtained from therapeutic immunoadsorption (containing high titers of aCL IgG antibodies and high titers of high-avidity anti- $\beta 2$ -GPI IgG antibodies) and was concentrated using Amicon ultracentrifugation cell with YM 100,000 membrane (Millipore) to a concentration of 52 mg/mL. The final concentration of the IgG fraction from an APS patient in the experiments ranged from 0.077–5.2 mg/mL. High-avidity polyclonal anti- $\beta 2$ -GPI IgG antibodies were isolated from a second APS patient IgG fraction by affinity purification on a CNBr-activated agarose (Sigma-Aldrich Chemie, Taufkirchen, Germany) column with bound pure unnicked  $\beta 2$ -GPI.<sup>30</sup> The antibodies were equilibrated against PBS and were concentrated using Amicon ultracentrifugation cell with YM 100,000 membrane (Millipore) to a concentration of 335  $\mu\text{g/mL}$ . The final concentration of polyclonal anti- $\beta 2$ -GPI IgG antibodies in the experiments ranged from 10.5–33.5  $\mu\text{g/mL}$ . HCAL, a chimeric monoclonal IgG antibody consisting of human  $\kappa$  and  $\gamma 1$  constant regions and variable regions from the mouse monoclonal antibody WBCAL (that has the specificity similar to aCL from APS patient sera),<sup>31,32</sup> was used at the concentration of 0.22  $\mu\text{g/mL}$ . The concentrations of antibodies and  $\beta 2$ -GPI were determined using Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with BSA as standard. LMWH used in the experiments was nadroparin calcium (anti-Xa 9500 IU/mL) (Fraxiparine®, Glaxo-SmithKline, Brentford, Middlesex, UK). The final concentration of nadroparin in the experiments was anti-Xa 1.2 IU/mL, which is within the therapeutic range of intravenous nadroparin concentration used for treatment of thromboembolic disorders. Before the addition into the GPV suspension,  $\beta 2$ -GPI  $\pm$  antibodies  $\pm$  nadroparin were preincubated for 10 min at room temperature.

### Experimental Procedure for GPV Characterization and Observation

Experiments were performed at room temperature in 70- $\mu\text{L}$  CoverWell™ perfusion chambers (Grace Bio-Labs, Bend, OR, USA) sealed to the microscope slide that allowed four experiments to be done in parallel. For the experiments, 18  $\mu\text{L}$  of sugar/PBS suspension containing GPVs was added into each perfusion chamber. As the GPVs settled down onto the microscope slide, 14  $\mu\text{L}$  of sugar/PBS solution containing test compound(s) ( $\beta 2$ -GPI, IgG antibodies from a healthy donor, IgG fraction from an APS patient, polyclonal anti- $\beta 2$ -GPI IgG antibodies, and/or nadroparin) was added to the GPV suspension. The sugar/PBS solution containing test compounds was of the same composition as the solution in which the GPVs were resuspended (0.14 mol/L glucose, 0.14 mol/L sucrose, 0.084 mol/L PBS). This ensured that, following the addition of the test solution, the background suspension of GPVs was not affected and that the only net change that occurred was the addition of test compound(s). As a background control, sugar/PBS solution containing 0.14 mol/L glucose, 0.14 mol/L sucrose, 0.084 mol/L PBS was used.

The adhesion between GPVs in the presence of test compound(s) was observed by using an inverted microscope Zeiss Axiovert 200 (Carl Zeiss MicroImaging, Jena, Germany) with phase-contrast optics and was recorded with a VisiCam 1280 camera (Visitron Systems, Puchheim, Germany). The images of adhered GPVs were acquired in the time interval of 25–30 min after the addition of the test solution, using the MetaMorph imaging system (Visitron). Under the phase contrast microscope GPVs containing sucrose solution appeared darker in comparison to the surrounding sucrose/glucose/PBS solution because of the differences in refraction indices of the solutions.

**TABLE 1.** Effect of Nadroparin on Adhesion between Negatively Charged Phosphatidylserine-Containing Giant Phospholipid Vesicles (POPS-GPVs) in the Presence of  $\beta 2$ -Glycoprotein ( $\beta 2$ -GPI)  $\pm$  Healthy Donor IgG Antibodies or Anti- $\beta 2$ -GPI IgG Antibodies from an Antiphospholipid Syndrome (APS) Patient

	Average effective angle of contact (degrees)		
	1st Batch of GPVs	2nd Batch of GPVs	3rd Batch of GPVs
$\beta 2$ -GPI	105 $\pm$ 20	93 $\pm$ 19	94 $\pm$ 16
$\beta 2$ -GPI + healthy donor IgG	110 $\pm$ 17	100 $\pm$ 24	91 $\pm$ 17
$\beta 2$ -GPI + anti- $\beta 2$ -GPI IgG	47 $\pm$ 11	64 $\pm$ 23	69 $\pm$ 23
Nadroparin	0	0	0
$\beta 2$ -GPI + nadroparin	93 $\pm$ 27	ND	ND
$\beta 2$ -GPI + healthy donor IgG + nadroparin	106 $\pm$ 20	93 $\pm$ 27	98 $\pm$ 23
$\beta 2$ -GPI + anti- $\beta 2$ -GPI IgG + nadroparin	ND	104 $\pm$ 30	95 $\pm$ 28
Healthy donor IgG	0	0	0
Anti- $\beta 2$ -GPI IgG	0	0	0
Healthy donor IgG + nadroparin	ND	0	0
Anti- $\beta 2$ -GPI IgG + nadroparin	ND	0	0
Background control	0	0	0

Experiments were done on three different batches of electroformation-obtained GPVs. The indicated concentrations were used:  $\beta 2$ -GPI (55  $\mu\text{g/mL}$ ), healthy donor IgG (33.6  $\mu\text{g/mL}$  in first batch and 42.1  $\mu\text{g/mL}$  in second and third batches), anti- $\beta 2$ -GPI IgG (33.5  $\mu\text{g/mL}$ ), nadroparin (anti-Xa 1.2 IU/mL). See text for statistical significance. ND, not determined.

Measurement of Adhesion between GPVs

The strength of adhesion between GPVs was determined semiquantitatively by measuring effective angles of contact between adhered GPVs<sup>26</sup> in the frames acquired 25–30 min after the addition of the test solution into the suspension of GPVs, using Image J software (National Institutes of Health, Bethesda, MD, <http://rsb.info.nih.gov/ij>). On average, 500 angles of contact between adhered GPVs were measured for each experiment, and the average effective angle of contact was calculated. The larger average effective angle of contact represents stronger adhesion between GPVs, while smaller average effective angle of contact represents weaker adhesion between GPVs.

Statistical Analysis

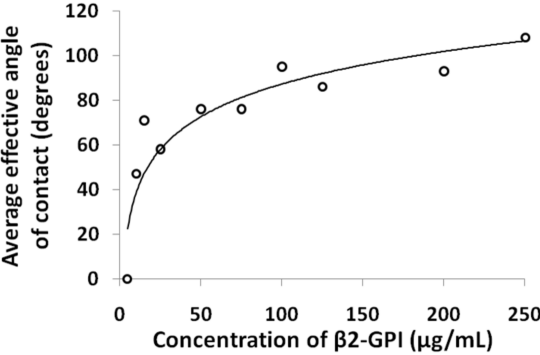
Statistical analysis was performed using SPSS 15.0 software (SPSS Inc., Chicago, IL). For the average effective angle of contact between GPVs, descriptive statistical parameters (average, standard deviation, frequencies,

frequency distribution) were calculated. Values of average effective angles of contact between GPVs in the presence of different test solutions were compared within each set of experiments using one-way ANOVA and *post hoc* multiple comparison analysis (Dunnett’s *C* test). Significance was defined as a 95% confidence interval.

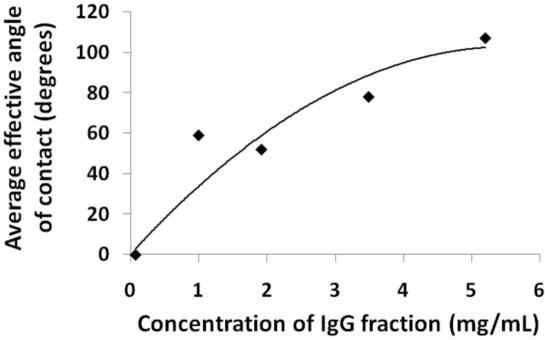
Results

Incubation of GPVs with  $\beta 2$ -GPI

The addition of  $\beta 2$ -GPI into the GPV suspension induced strong adhesion between negatively charged POPS-GPVs, with average effective angles of contact ranging from 93–105° (Table 1), while no adhesion between neutral GPVs could be observed (data not shown). The adhesion between POPS-GPVs in the presence of  $\beta 2$ -GPI was concentration dependent (Fig. 1). There was no adhesion between POPS-GPVs at  $\beta 2$ -GPI concentration as low as 4.6  $\mu\text{g/mL}$ , while near maximal adhesion (93°) was reached within the physiological range of  $\beta 2$ -GPI concentration (200  $\mu\text{g/mL}$ ).



**Figure 1.** The adhesion between negatively charged phosphatidylserine-containing giant phospholipid vesicles (POPS-GPVs) in the presence of increasing concentrations of  $\beta 2$ -glycoprotein ( $\beta 2$ -GPI).

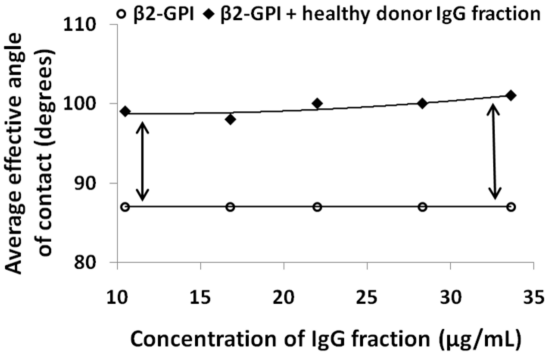


**Figure 2.** The adhesion between POPS-GPVs in the presence of increasing concentrations of IgG fraction from an antiphospholipid syndrome (APS) patient containing high titers of anticardiolipin antibodies (aCL) and high-avidity anti- $\beta 2$ -GPI antibodies.

### Incubation of GPVs with Polyclonal IgG Antibodies or Monoclonal IgG Antibody HCAL

Healthy donor IgG antibodies and polyclonal anti- $\beta 2$ -GPI IgG antibodies from an APS patient at the concentrations ranging from 10.5–77  $\mu\text{g/mL}$  did not induce adhesion between POPS-GPVs (some shown by zero values of average effective angles of contact between GPVs in Table 1). There was no adhesion between POPS-GPVs in the presence of an IgG fraction from a syphilitic patient (77  $\mu\text{g/mL}$ ) and a monoclonal IgG antibody HCAL (0.22  $\mu\text{g/mL}$ ) (data not shown). Also, no adhesion between POPS-GPVs could be observed when PBS/sugar solution alone was added into the GPV suspension (Table 1).

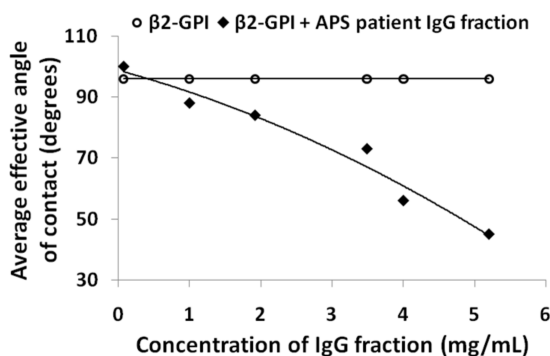
However, when POPS-GPVs were incubated with larger concentrations ( $\geq 1$  mg/mL) of an APS IgG fraction containing high titers of both aCL and anti- $\beta 2$ -GPI antibodies, a dose-dependent increase in the adhesion of POPS-GPVs was observed (Fig. 2). The average effective angles of contact increased from 0° at antibody concentration of 0.077 mg/mL to 107° at antibody concentration of 5.2 mg/mL, which is approximately half of the IgG antibody concentration in human plasma (Fig. 2).



**Figure 3.** The changes in adhesion between POPS-GPVs in the presence of  $\beta 2$ -GPI (55  $\mu\text{g/mL}$ ) and increasing concentrations of IgG antibodies from a healthy donor serum. The arrows indicate the increase in adhesion when healthy donor IgG antibodies are added, emphasizing comparison between points.

### Incubation of GPVs in the Presence of $\beta 2$ -GPI and IgG Antibodies from a Healthy Donor

In the presence of IgG antibodies from a healthy donor, a statistically significant increase in  $\beta 2$ -GPI-induced adhesion between POPS-GPVs was observed in seven out of eight experiments done on four different batches of electroformation-obtained GPVs. The average effective angle of contact between POPS-GPVs increased from 105–110° (first batch, Table 1), from 93–100° (second batch, Table 1), and from 87° to approximately 100° (fourth batch, Fig. 3),

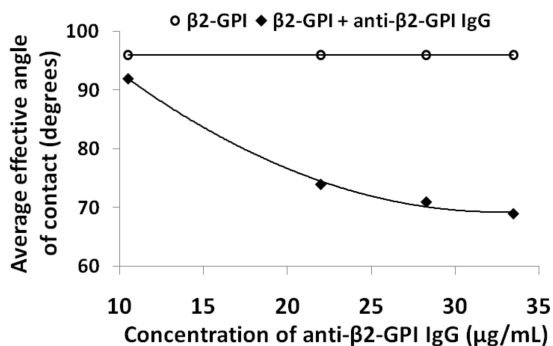


**Figure 4.** The reduction of  $\beta$ 2-GPI-induced adhesion between POPS-GPVs in the presence of increasing concentrations of IgG fraction from an APS patient containing high titers of aCL and high-avidity anti- $\beta$ 2-GPI antibodies.

while in one batch a small statistically nonsignificant decrease in adhesion between POPS-GPVs was observed (third batch, Table 1). A statistically significant rise in membrane adhesion in the presence of  $\beta$ 2-GPI was observed with a healthy donor IgG antibody concentration as low as 10.6  $\mu$ g/mL. There was no further substantial increase in membrane adhesion when the antibody concentration was elevated to 33.6  $\mu$ g/mL (Fig. 3).

#### Reduction of Adhesion between GPVs by IgG Fraction from an APS Patient

In the presence of the increasing concentrations of IgG fraction from an APS patient (containing high titers of both aCL and high-avidity anti- $\beta$ 2-GPI antibodies),  $\beta$ 2-GPI-induced adhesion between POPS-GPVs was reduced in a dose-dependent manner (Fig. 4). A statistically significant reduction of  $\beta$ 2-GPI-induced adhesion (8.5%) between POPS-GPVs was reached at the antibody concentration of 1 mg/mL. At the antibody concentration of 5.2 mg/mL,  $\beta$ 2-GPI-induced adhesion between POPS-GPVs decreased by more than 50%. The rise in  $\beta$ 2-GPI-induced adhesion from 96–100° at the antibody concentration of 0.077 mg/mL was not statistically significant (Fig. 4).



**Figure 5.** The reduction of  $\beta$ 2-GPI-induced adhesion between POPS-GPVs in the presence of increasing concentrations of anti- $\beta$ 2-GPI IgG antibodies from an APS patient serum.

#### Reduction of Adhesion between GPVs by Polyclonal anti- $\beta$ 2-GPI Antibodies and Monoclonal Antibody HCAL

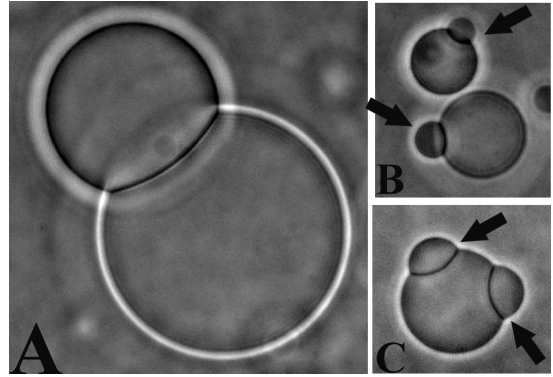
In contrast to IgG antibodies from a healthy donor, the same concentrations of high-avidity polyclonal anti- $\beta$ 2-GPI IgG antibodies from an APS patient greatly reduced  $\beta$ 2-GPI-induced adhesion of POPS-GPVs. The reduction of  $\beta$ 2-GPI-induced adhesion of POPS-GPVs was statistically significant, with average effective angles of contact decreasing from 105°, 93°, and 94° to 47°, 64°, and 69°, respectively (Table 1). A proportion of GPVs did not adhere at all. In the presence of increasing concentrations of polyclonal anti- $\beta$ 2-GPI IgG antibodies,  $\beta$ 2-GPI-induced adhesion between POPS-GPVs was reduced in a dose-dependent manner (Fig. 5). A statistically significant decrease in  $\beta$ 2-GPI-induced adhesion of POPS-GPVs was reached at an anti- $\beta$ 2-GPI IgG concentration of 22  $\mu$ g/mL. Preincubation of  $\beta$ 2-GPI and healthy donor IgG antibodies with high-avidity polyclonal anti- $\beta$ 2-GPI IgG antibodies induced a large and statistically significant reduction in the adhesion of POPS-GPVs (data not shown). The monoclonal IgG antibody HCAL significantly reduced  $\beta$ 2-GPI-induced adhesion between POPS-GPVs at a concentration as low as 0.22  $\mu$ g/mL (data not shown).

### The Rescue of Reduced Adhesion between GPVs by Nadroparin

Nadroparin in therapeutic concentration reduced  $\beta$ 2-GPI-induced adhesion between POPS-GPVs, with the average effective angle of contact between GPVs decreasing from 105–93° (first batch in Table 1). Although the decrease in  $\beta$ 2-GPI-induced membrane adhesion was statistically significant, the average effective angle of contact between GPVs remained rather large (93°) compared to only 47° with anti- $\beta$ 2-GPI IgG antibodies.

$\beta$ 2-GPI-induced membrane adhesion between POPS-GPVs was larger in the presence of IgG antibodies from a healthy donor (110°) than in the presence of nadroparin (93°), and the difference was statistically significant (Table 1). Preincubation of  $\beta$ 2-GPI and IgG antibodies from a healthy donor with nadroparin resulted in a statistically significant reduction of membrane adhesion between POPS-GPVs in two out of three batches of electroformation-obtained GPVs (first and second batches in Table 1), while in the third batch a nonsignificant increase in membrane adhesion was observed. Irrespective of the significant decrease in  $\beta$ 2-GPI-induced membrane adhesion, average effective angles of contact remained rather large (106° and 93°) and did not differ significantly from membrane adhesion observed in the presence of  $\beta$ 2-GPI alone (105° and 93°).

The therapeutic concentration of nadroparin (anti-Xa 1.2 IU/mL) completely restored (rescued)  $\beta$ 2-GPI-induced adhesion of POPS-GPVs that was significantly reduced in the presence of anti- $\beta$ 2-GPI IgG antibodies from an APS patient. The observed rise in POPS-GPV adhesion was statistically significant, with the average effective angles of contact increasing from 64° and 69° to 104° and 95°, respectively. In the presence of anti- $\beta$ 2-GPI IgG antibodies, the direct negative effect of nadroparin on  $\beta$ 2-GPI-induced membrane adhesion did not seem to be present because the level of membrane adhesion was the same (95°) or larger (104°) than in the presence of  $\beta$ 2-GPI alone



**Figure 6.** Adhesion between POPS-GPVs (A) with strong adhesion of buds to mother vesicle membrane (arrows in B, C) in the presence of nadroparin (anti-Xa 8.9 IU/mL).

(94° and 93°, respectively). This lack of the inhibitory effect of nadroparin on  $\beta$ 2-GPI membrane binding might contribute further to the rescue of membrane adhesion in the presence of nadroparin. Also, membrane adhesion in the presence of  $\beta$ 2-GPI, anti- $\beta$ 2-GPI IgG antibodies, and nadroparin did not differ significantly from the level of membrane adhesion observed with  $\beta$ 2-GPI and healthy donor IgG antibodies (91° and 100°, respectively). Data are shown in Table 1.

Nadroparin alone in the therapeutic concentration did not induce adhesion between POPS-GPVs (zero values of average effective angle of contact shown in Table 1). However, with larger concentrations of nadroparin (anti-Xa 8.9 IU/mL and 178 IU/mL) a concentration-dependent increase in adhesion of POPS-GPVs was found and was accompanied with strong adhesion of buds to the mother vesicle (Fig. 6).

### Discussion

Several mechanisms were proposed to explain the therapeutic effects of heparins (unfractionated and LMWHs) in the treatment of acute thrombosis and pregnancy complications in APS patients. Heparins were found to directly affect the coagulation cascade, to



inhibit the binding of  $\beta 2$ -GPI to negatively charged phospholipids,<sup>4</sup> to promote plasmin-mediated cleavage of  $\beta 2$ -GPI,<sup>4</sup> to directly inhibit aPL binding to negatively charged phospholipids in aPL ELISA,<sup>5,6</sup> and to enhance clearance of aPL *in vivo*.<sup>7,8</sup> Regarding pregnancy complications, heparins were reported to inhibit aPL binding to trophoblast cells, to promote trophoblast invasiveness, to modulate trophoblast apoptosis, and to inhibit complement cascade activation (as reviewed in Ref. 9). However, the role of heparin in the modulation of membrane microvesiculation, which is increasingly appreciated to contribute to the hypercoagulability in APS, is not yet understood.

To investigate the potential role of a therapeutic concentration of LMWH in the processes of membrane adhesion and vesiculation in APS, the interactions between  $\beta 2$ -GPI, polyclonal anti- $\beta 2$ -GPI IgG antibodies from an APS patient, APS patient IgG fraction, healthy donor IgG antibodies, and nadroparin were studied in a GPV model. GPVs represent a valuable *in vitro* membrane model to study adhesion between phospholipid membranes. Because of their size (20–100  $\mu\text{m}$ ), GPVs mimic more closely physiological properties of cell membranes<sup>33</sup> and enable a direct observation of membrane adhesion under optical microscopy. However, there have been some limitations encountered. Specifically, the quantity of GPVs from a single electroformation is limited and some variability in properties of GPVs from multiple electroformations is expected. The absolute values of average effective angles of contact can, therefore, be directly compared only within the set of experiments done on the same batch of GPVs. This is why our experiments were performed on multiple batches of electroformed GPVs.

Polyclonal anti- $\beta 2$ -GPI IgG antibodies from an APS patient as well as an IgG fraction from a second APS patient (containing high titers of aCL and anti- $\beta 2$ -GPI antibodies) but not IgG antibodies from a healthy donor, significantly reduced  $\beta 2$ -GPI-induced adhesion between

negatively charged POPS-GPVs. Meanwhile, preincubation of  $\beta 2$ -GPI and anti- $\beta 2$ -GPI IgG antibodies with the therapeutic concentration of nadroparin completely restored  $\beta 2$ -GPI-induced membrane adhesion.

### $\beta 2$ -GPI, IgG Antibodies, and GPV Interactions

The addition of a physiological concentration of  $\beta 2$ -GPI into the GPV suspension induced strong adhesion between negatively charged POPS-GPVs, while no adhesion between neutral GPVs was observed. This is consistent with previous reports that negatively charged phospholipids are essential for membrane binding of  $\beta 2$ -GPI (as reviewed in Ref. 34) and may be of functional importance for the role of  $\beta 2$ -GPI in the prevention of membrane vesiculation. Namely, the exposure of negatively charged phospholipids on platelet membrane surfaces was shown to precede membrane microvesiculation.<sup>35</sup>

Anti- $\beta 2$ -GPI IgG antibodies might contribute strongly to reduction of  $\beta 2$ -GPI-induced membrane adhesion in APS patients (Figs. 4 and 5). Specifically, the IgG fraction from an APS patient (containing high titers of aCL and anti- $\beta 2$ -GPI antibodies) reduced  $\beta 2$ -GPI-induced membrane adhesion by more than 50% at near physiological IgG antibody to the free  $\beta 2$ -GPI ratio as seen in human plasma (Fig. 4). Moreover, a statistically significant reduction of  $\beta 2$ -GPI-induced adhesion was observed at the APS IgG fraction concentration of 1 mg/mL, where the ratio between the IgG antibodies and free  $\beta 2$ -GPI was fourfold smaller than in human plasma (Fig. 4). A direct role of anti- $\beta 2$ -GPI IgG antibodies in decreasing  $\beta 2$ -GPI-induced membrane adhesion was confirmed by preincubation of  $\beta 2$ -GPI and healthy donor IgG antibodies with anti- $\beta 2$ -GPI IgG antibodies (data not shown). The reduction of  $\beta 2$ -GPI-induced membrane adhesion in the presence of anti- $\beta 2$ -GPI IgG antibodies is most probably a result of the interference of anti- $\beta 2$ -GPI antibodies with membrane binding of

domain I of  $\beta 2$ -GPI. Domain I of  $\beta 2$ -GPI was shown to be involved in aggregation/precipitation of negatively charged vesicles.<sup>36,37</sup> Anti- $\beta 2$ -GPI IgG antibodies might bind domain I directly or may sterically hinder its interaction with the membrane.

In contrast to anti- $\beta 2$ -GPI IgG antibodies, IgG antibodies from a healthy donor significantly increased  $\beta 2$ -GPI-induced membrane adhesion of POPS-GPVs. This might be a result of the nonspecific cross-linking of juxtaposed negatively charged GPV membranes by a proportion of IgG antibodies having positively charged paratopes. Further confirmation of this mechanism was obtained from the incubation of POPS-GPVs with  $\beta 2$ -GPI and a syphilitic IgG fraction that contained only high titers of aCL antibodies (data not shown). It could be inferred that in healthy individuals IgG antibodies at concentrations as low as 10.6  $\mu\text{g/mL}$  potentially enhance the role of  $\beta 2$ -GPI in regulating membrane adhesion and vesiculation. Moreover, a strong adhesion between membranes ( $107^\circ$ ) was observed when POPS-GPVs were incubated with an IgG fraction from an APS patient at the concentration of 5.2 mg/mL (approximately half the concentration of IgG antibodies in plasma) (Fig. 2). Based on this observation two conclusions can be made. First, the pathogenic effect of anti- $\beta 2$ -GPI antibodies on membrane adhesion would occur only if  $\beta 2$ -GPI is simultaneously present in the solution (as compared in Figs. 2 and 4). And second, in the absence of  $\beta 2$ -GPI, the near physiological concentration of IgG antibodies may not only supplement but could also effectively replace the role of  $\beta 2$ -GPI in membrane adhesion (Fig. 2). The latter could at least, in part, explain why the deficiency of  $\beta 2$ -GPI does not lead to thrombosis.

### The Effect of Nadroparin on Protein–GPV Interactions

Nadroparin itself in the therapeutic concentration did not induce adhesion of negatively charged POPS-GPVs. However,

with larger concentrations of nadroparin, a dose-dependent adhesion between negatively charged POPS-GPVs was observed along with the strong adhesion of buds to the mother vesicle membrane (Fig. 6).

Preincubation of  $\beta 2$ -GPI, as well as  $\beta 2$ -GPI plus healthy donor IgG antibodies, with nadroparin, significantly decreased  $\beta 2$ -GPI-induced adhesion of negatively charged POPS-GPVs. This is most probably a result of the interference of nadroparin with membrane binding of domain V of  $\beta 2$ -GPI.  $\beta 2$ -GPI was shown to bind heparin either immobilized on sepharose columns<sup>38</sup>/Nunc Maxisorp plates<sup>4</sup> or in fluid phase.<sup>4</sup> Moreover, using expression/site-directed mutagenesis studies of  $\beta 2$ -GPI binding to heparin-coated plates, it was shown that the primary heparin-binding site resides within the major phospholipid-binding site on domain V of  $\beta 2$ -GPI.<sup>4</sup> Irrespective of the significant decrease in  $\beta 2$ -GPI-induced membrane adhesion in the presence of nadroparin, the average effective angle of contact between POPS-GPVs remained rather large ( $93^\circ$ ), indicating that only a small proportion of  $\beta 2$ -GPI molecules interacted with nadroparin. This is consistent with a small decrease in aPL binding on phospholipid-coated microtiter plates in cofactor-( $\beta 2$ -GPI)-dependent phosphatidylserine and cardiolipin ELISA after preincubation of adult bovine serum with heparin.<sup>5</sup>

Therapeutic concentration of nadroparin completely restored (rescued)  $\beta 2$ -GPI-induced adhesion between negatively charged POPS-GPVs in the presence of high-avidity polyclonal anti- $\beta 2$ -GPI IgG antibodies, which points to a possible role of nadroparin in the modulation of membrane microvesiculation in APS. Moreover, after preincubation of  $\beta 2$ -GPI and anti- $\beta 2$ -GPI antibodies with nadroparin, membrane adhesion did not differ significantly from the level of membrane adhesion observed with  $\beta 2$ -GPI and healthy donor IgG antibodies (Table 1). This implies that nadroparin rescued the adhesion between membranes to such a level that is comparable to the one seen in physiological conditions.

Nadroparin most probably interferes with anti- $\beta$ 2-GPI antibody binding to membrane-bound  $\beta$ 2-GPI, enabling domain I of  $\beta$ 2-GPI to freely interact with the negatively charged membranes. This is consistent with the inhibition of *in vitro* binding of aPL on phospholipid-coated microplates in  $\beta$ 2-GPI-dependent phosphatidylserine and cardiolipin ELISA in the presence of LMWH or unfractionated heparin.<sup>5–7</sup> Further, affinity chromatography with unfractionated heparin and LMWH columns adsorbed a significant proportion of aPL from sera of APS women with recurrent pregnancy loss.<sup>6,7</sup>

In general, heparin–protein interaction(s) are primarily ionic in nature—they are a result of the binding of negatively charged sulfo- and carboxyl- groups on heparin to positively charged amino acids on the protein.<sup>39</sup> Hydrogen bonds are also important, at least in some cases of protein–heparin interaction.<sup>39</sup> The interacting groups within heparin and proteins must be appropriately positioned and oriented to confer the specificity of heparin–protein interactions.<sup>39</sup> A distinguishing feature of IgG aPL antibodies are somatic mutations that lead to accumulation of positively charged amino acids (arginine, asparagine, and lysine) within the complementary-determining regions of the paratope.<sup>40</sup> Further, arginine residues were implicated in binding of human monoclonal aPL derived from an APS patient to  $\beta$ 2-GPI.<sup>41</sup> Based on these observations it could be inferred that nadroparin might potentially bind positively charged amino acids within the paratope of aPL (through ionic interactions and/or hydrogen bonds), preventing their interaction with  $\beta$ 2-GPI.

An additional mechanism might exist through which nadroparin restores membrane adhesion in the presence of anti- $\beta$ 2-GPI antibodies. An increase in the surface density of the negative charge within POPS-GPVs might be expected in the presence of nadroparin because heparin was reported to selectively strip off phosphatidylcholine from phospholipid vesicles.<sup>42</sup> This could further increase  $\beta$ 2-GPI-

induced adhesion between POPS-GPVs and could at least partly explain the significantly larger membrane adhesion in the presence of  $\beta$ 2-GPI, anti- $\beta$ 2-GPI IgG, and nadroparin than in the presence of  $\beta$ 2-GPI alone (second batch of GPVs in Table 1).

In conclusion, enhanced microvesiculation of cell membranes with dissemination of procoagulant membrane properties is increasingly accepted as one of the mechanisms contributing to the hypercoagulability in APS. The decrease in  $\beta$ 2-GPI-induced membrane adhesion in the presence of anti- $\beta$ 2-GPI antibodies suggests a direct role of anti- $\beta$ 2-GPI antibodies in enhancing membrane microvesiculation and is consistent with the finding that anti- $\beta$ 2-GPI antibodies directed against domain I strongly correlate with thrombosis in APS.<sup>43</sup> In the presence of anti- $\beta$ 2-GPI IgG antibodies, nadroparin completely restored the interactions between negatively charged phospholipid membranes and  $\beta$ 2-GPI in the GPV model that mimics more closely the physiological properties of phospholipid membranes. A novel anticoagulant mechanism of nadroparin is suggested that supplements its direct effect on the coagulation cascade. Restoration of adhesion between negatively charged membranes in the presence of nadroparin might decrease shedding of microvesicles into surrounding solution and might contribute to efficacy of heparin in the treatment of acute thrombosis and pregnancy complications in APS.

In this contribution we were unable to summarize  $\beta$ 2-GPI structural and functional details as well as the most current issues related to associations between aPL and microvesiculation. For this reason, we refer to recent literature on these subjects.<sup>44–47</sup>

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### Conflicts of Interest

The authors declare no conflicts of interest.

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