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 β2-glycoprotein I (β2-GPI)-induced membrane adhesion, indicating that IgG antibodies might supplement the role of β2-GPI in the regulation of membrane microvesiculation in healthy individuals. Anti-β2-GPI IgG antibodies significantly reduced β2-GPI-induced membrane adhesion, suggesting a direct role of anti-β2-GPI antibodies in enhancing membrane microvesiculation in APS. Therapeutic concentration of nadroparin completely restored β2-GPI-induced membrane adhesion in the presence of anti-β2-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-β2-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.[1] 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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Contemporary Challenges in Autoimmunity: Ann. N.Y. Acad. Sci. 1173: 874–886 (2009).
doi: 10.1111/j.1749-6632.2009.04745.x

are treated with standard heparin plus aspirin, and similar live-birth rates could be achieved with LMWHs.[2,3] 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.[4–9]

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[10] 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.[11] Increased levels of circulating endothelial-[10,12–14] as well as platelet-derived[12] 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.[15] 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.[16] Treatment of pregnant mice with phosphatidylserine-containing phospholipid vesicles induced placental vessel thrombosis and led to intrauterine growth restriction.[17]

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.[10] In an *in vitro* model of a single giant phospholipid vesicle (GPV), anti-β2-glycoprotein I (anti-β2-GPI) antibodies from an APS patient in the presence of β2-GPI directly enhanced budding of the negatively charged GPV membrane and induced membrane vesiculation.[19]

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.[20] According to the hypothesis, attractive interactions between phospholipid membranes in the presence of certain plasma proteins, especially β2-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.[20] 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.[21–23] 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 β2-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.[24] 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)[25] 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[26] as well as from gastrointestinal patients)[27] and the strength of adhesion between phospholipid membranes after the addition of the plasma samples.

β2-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-β2-GPI antibodies to membrane-bound β2-GPI might importantly interfere with β2-GPI-induced adhesion between membranes, resulting in an increased microvesiculation in APS. In the present work the interactions between the phospholipid membrane, β2-GPI, healthy donor IgG antibodies, IgG fraction from an APS patient, purified anti-β2-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.*[28]), as described in Janša *et al.*[27] After the electroformation, 600 μL 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 KH_2PO_4, 7.8 mmol/L $Na_2HPO_4 \cdot 2H_2O$, 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.

β2-GPI, IgG Antibodies, and Nadroparin

β2-GPI was isolated from pooled human plasma by a slightly modified method, as described previously,[29] 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 β2-GPI in PBS (5.9 mg/mL) were stored at –20°C. The final concentration of β2-GPI in the experiments was 55 μg/mL, which is approximately half of free β2-GPI concentration in human plasma. In the experiments where the influence of β2-GPI concentration on membrane adhesion was tested, the final concentrations of β2-GPI ranged from 4.6–250 μg/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 μg/mL, and the final concentration of the syphilitic IgG fraction was 77 μg/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-β2-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-β2-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 β2-GPI.[30] The antibodies were equilibrated against PBS and were concentrated using Amicon ultracentrifugation cell with YM 100,000 membrane (Millipore) to a concentration of 335 μg/mL. The final concentration of polyclonal anti-β2-GPI IgG antibodies in the experiments ranged from 10.5–33.5 μg/mL. HCAL, a chimeric monoclonal IgG antibody consisting of human κ and γ1 constant regions and variable regions from the mouse monoclonal antibody WBCAL (that has the specificity similar to aCL from APS patient sera),[31,32] was used at the concentration of 0.22 μg/mL. The concentrations of antibodies and β2-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®, GlaxoSmithKline, 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, β2-GPI ± antibodies ± nadroparin were preincubated for 10 min at room temperature.

Experimental Procedure for GPV Characterization and Observation

Experiments were performed at room temperature in 70-μL 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 μL of sugar/PBS suspension containing GPVs was added into each perfusion chamber. As the GPVs settled down onto the microscope slide, 14 μL of sugar/PBS solution containing test compound(s) (β2-GPI, IgG antibodies from a healthy donor, IgG fraction from an APS patient, polyclonal anti-β2-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, Pucheim, 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 β2-Glycoprotein (β2-GPI) ± Healthy Donor IgG Antibodies or Anti-β2-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
β2-GPI	105 ± 20	93 ± 19	94 ± 16
β2-GPI + healthy donor IgG	110 ± 17	100 ± 24	91 ± 17
β2-GPI + anti-β2-GPI IgG	47 ± 11	64 ± 23	69 ± 23
Nadroparin	0	0	0
β2-GPI + nadroparin	93 ± 27	ND	ND
β2-GPI + healthy donor IgG + nadroparin	106 ± 20	93 ± 27	98 ± 23
β2-GPI + anti-β2-GPI IgG + nadroparin	ND	104 ± 30	95 ± 28
Healthy donor IgG	0	0	0
Anti-β2-GPI IgG	0	0	0
Healthy donor IgG + nadroparin	ND	0	0
Anti-β2-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: β2-GPI (55 μg/mL), healthy donor IgG (33.6 μg/mL in first batch and 42.1 μg/mL in second and third batches), anti-β2-GPI IgG (33.5 μg/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[26] 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 β2-GPI

The addition of β2-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 β2-GPI was concentration dependent (Fig. 1). There was no adhesion between POPS-GPVs at β2-GPI concentration as low as 4.6 μg/mL, while near maximal adhesion (93°) was reached within the physiological range of β2-GPI concentration (200 μg/mL).

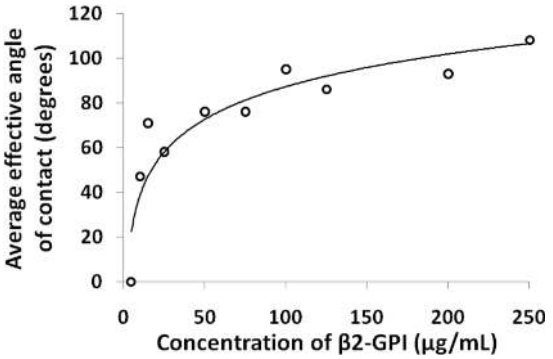

Figure 1. The adhesion between negatively charged phosphatidylserine-containing giant phospholipid vesicles (POPS-GPVs) in the presence of increasing concentrations of β2-glycoprotein (β2-GPI).

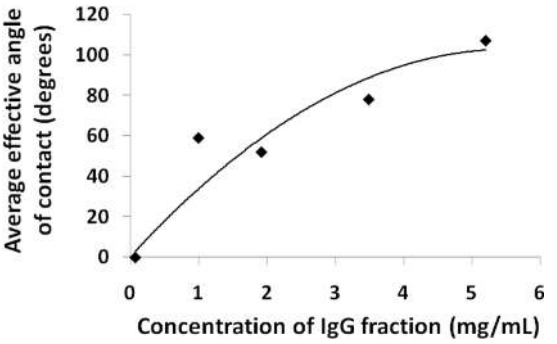

Figure 2. The adhesion between POPS-GPVs in the presence of increasing concentrations of IgG fraction from an antiphospholipid syndrome (APS) patient containing high titers of anticardiolipin antibodies (aCL) and high-avidity anti-β2-GPI antibodies.

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

Healthy donor IgG antibodies and polyclonal anti-β2-GPI IgG antibodies from an APS patient at the concentrations ranging from 10.5–77 μg/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 μg/mL) and a monoclonal IgG antibody HCAL (0.22 μg/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 (≥ 1 mg/mL) of an APS IgG fraction containing high titers of both aCL and anti-β2-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).

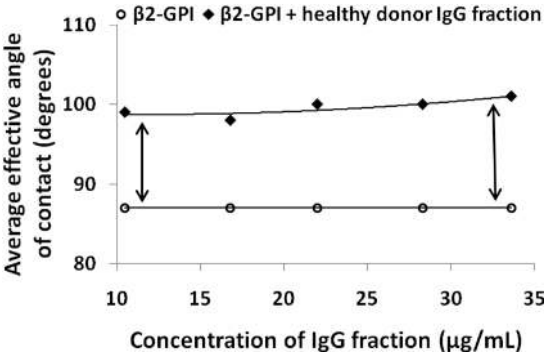

Figure 3. The changes in adhesion between POPS-GPVs in the presence of β2-GPI (55 μg/mL) and increasing concentrations of IgG antibodies from a healthy donor serum. The arrows indicate the increase in adhesion when healthy donor IgG antibodies are added, emphasizing comparison between points.

Incubation of GPVs in the Presence of β2-GPI and IgG Antibodies from a Healthy Donor

In the presence of IgG antibodies from a healthy donor, a statistically significant increase in β2-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),

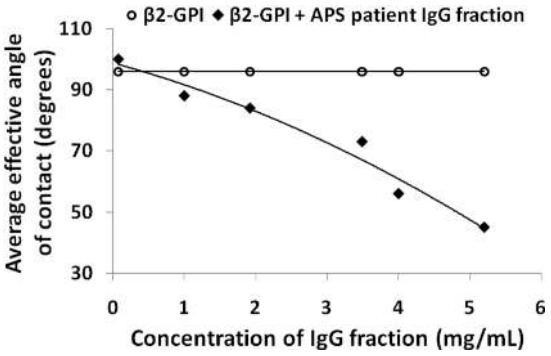

Figure 4. The reduction of β2-GPI-induced adhesion between POPS-GPVs in the presence of increasing concentrations of IgG fraction from an APS patient containing high titers of aCL and high-avidity anti-β2-GPI antibodies.

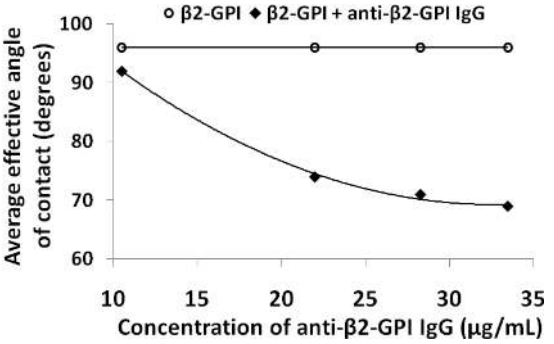

Figure 5. The reduction of β2-GPI-induced adhesion between POPS-GPVs in the presence of increasing concentrations of anti-β2-GPI IgG antibodies from an APS patient serum.

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 β2-GPI was observed with a healthy donor IgG antibody concentration as low as 10.6 μg/mL. There was no further substantial increase in membrane adhesion when the antibody concentration was elevated to 33.6 μg/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-β2-GPI antibodies), β2-GPI-induced adhesion between POPS-GPVs was reduced in a dose-dependent manner (Fig. 4). A statistically significant reduction of β2-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, β2-GPI-induced adhesion between POPS-GPVs decreased by more than 50%. The rise in β2-GPI-induced adhesion from 96–100° at the antibody concentration of 0.077 mg/mL was not statistically significant (Fig. 4).

Reduction of Adhesion between GPVs by Polyclonal anti-β2-GPI Antibodies and Monoclonal Antibody HCAL

In contrast to IgG antibodies from a healthy donor, the same concentrations of high-avidity polyclonal anti-β2-GPI IgG antibodies from an APS patient greatly reduced β2-GPI-induced adhesion of POPS-GPVs. The reduction of β2-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-β2-GPI IgG antibodies, β2-GPI-induced adhesion between POPS-GPVs was reduced in a dose-dependent manner (Fig. 5). A statistically significant decrease in β2-GPI-induced adhesion of POPS-GPVs was reached at an anti-β2-GPI IgG concentration of 22 μg/mL. Preincubation of β2-GPI and healthy donor IgG antibodies with high-avidity polyclonal anti-β2-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 β2-GPI-induced adhesion between POPS-GPVs at a concentration as low as 0.22 μg/mL (data not shown).

The Rescue of Reduced Adhesion between GPVs by Nadroparin

Nadroparin in therapeutic concentration reduced β2-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 β2-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-β2-GPI IgG antibodies.

β2-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 β2-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 β2-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 β2-GPI alone (105° and 93°).

The therapeutic concentration of nadroparin (anti-Xa 1.2 IU/mL) completely restored (rescued) β2-GPI-induced adhesion of POPS-GPVs that was significantly reduced in the presence of anti-β2-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-β2-GPI IgG antibodies, the direct negative effect of nadroparin on β2-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 β2-GPI alone (94° and 93°, respectively). This lack of the inhibitory effect of nadroparin on β2-GPI membrane binding might contribute further to the rescue of membrane adhesion in the presence of nadroparin. Also, membrane adhesion in the presence of β2-GPI, anti-β2-GPI IgG antibodies, and nadroparin did not differ significantly from the level of membrane adhesion observed with β2-GPI and healthy donor IgG antibodies (91° and 100°, respectively). Data are shown in Table 1.

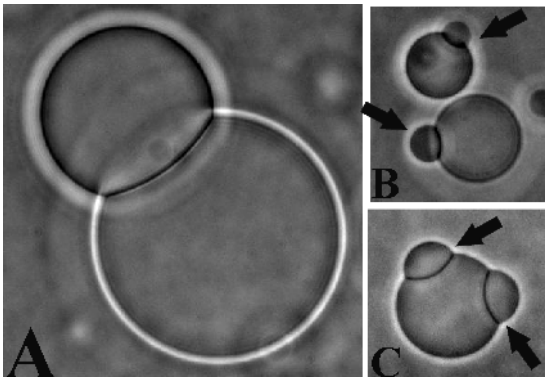

Figure 6. Adhesion between POPS-GPVs (**A**) with strong adhesion of buds to mother vesicle membrane (arrows in **B**, **C**) in the presence of nadroparin (anti-Xa 8.9 IU/mL).

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 β2-GPI to negatively charged phospholipids,[4] to promote plasmin-mediated cleavage of β2-GPI,[4] to directly inhibit aPL binding to negatively charged phospholipids in aPL ELISA,[5,6] and to enhance clearance of aPL *in vivo*.[7,8] 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 β2-GPI, polyclonal anti-β2-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 μm), GPVs mimic more closely physiological properties of cell membranes[33] 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-β2-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-β2-GPI antibodies) but not IgG antibodies from a healthy donor, significantly reduced β2-GPI-induced adhesion between negatively charged POPS-GPVs. Meanwhile, preincubation of β2-GPI and anti-β2-GPI IgG antibodies with the therapeutic concentration of nadroparin completely restored β2-GPI-induced membrane adhesion.

β2-GPI, IgG Antibodies, and GPV Interactions

The addition of a physiological concentration of β2-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 β2-GPI (as reviewed in Ref. 34) and may be of functional importance for the role of β2-GPI in the prevention of membrane vesiculation. Namely, the exposure of negatively charged phospholipids on platelet membrane surfaces was shown to precede membrane microvesiculation.[35]

Anti-β2-GPI IgG antibodies might contribute strongly to reduction of β2-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-β2-GPI antibodies) reduced β2-GPI-induced membrane adhesion by more than 50% at near physiological IgG antibody to the free β2-GPI ratio as seen in human plasma (Fig. 4). Moreover, a statistically significant reduction of β2-GPI-induced adhesion was observed at the APS IgG fraction concentration of 1 mg/mL, where the ratio between the IgG antibodies and free β2-GPI was fourfold smaller than in human plasma (Fig. 4). A direct role of anti-β2-GPI IgG antibodies in decreasing β2-GPI-induced membrane adhesion was confirmed by preincubation of β2-GPI and healthy donor IgG antibodies with anti-β2-GPI IgG antibodies (data not shown). The reduction of β2-GPI-induced membrane adhesion in the presence of anti-β2-GPI IgG antibodies is most probably a result of the interference of anti-β2-GPI antibodies with membrane binding of

domain I of β2-GPI. Domain I of β2-GPI was shown to be involved in aggregation/precipitation of negatively charged vesicles.[36,37] Anti-β2-GPI IgG antibodies might bind domain I directly or may sterically hinder its interaction with the membrane.

In contrast to anti-β2-GPI IgG antibodies, IgG antibodies from a healthy donor significantly increased β2-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 β2-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 μg/mL potentially enhance the role of β2-GPI in regulating membrane adhesion and vesiculation. Moreover, a strong adhesion between membranes (107°) 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-β2-GPI antibodies on membrane adhesion would occur only if β2-GPI is simultaneously present in the solution (as compared in Figs. 2 and 4). And second, in the absence of β2-GPI, the near physiological concentration of IgG antibodies may not only supplement but could also effectively replace the role of β2-GPI in membrane adhesion (Fig. 2). The latter could at least, in part, explain why the deficiency of β2-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 β2-GPI, as well as β2-GPI plus healthy donor IgG antibodies, with nadroparin, significantly decreased β2-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 β2-GPI. β2-GPI was shown to bind heparin either immobilized on sepharose columns[38]/Nunc Maxisorp plates[4] or in fluid phase.[4] Moreover, using expression/site-directed mutagenesis studies of β2-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 β2-GPI.[4] Irrespective of the significant decrease in β2-GPI-induced membrane adhesion in the presence of nadroparin, the average effective angle of contact between POPS-GPVs remained rather large (93°), indicating that only a small proportion of β2-GPI molecules interacted with nadroparin. This is consistent with a small decrease in aPL binding on phospholipid-coated microtiter plates in cofactor-(β2-GPI)-dependent phosphatidylserine and cardiolipin ELISA after preincubation of adult bovine serum with heparin.[5]

Therapeutic concentration of nadroparin completely restored (rescued) β2-GPI-induced adhesion between negatively charged POPS-GPVs in the presence of high-avidity polyclonal anti-β2-GPI IgG antibodies, which points to a possible role of nadroparin in the modulation of membrane microvesiculation in APS. Moreover, after preincubation of β2-GPI and anti-β2-GPI antibodies with nadroparin, membrane adhesion did not differ significantly from the level of membrane adhesion observed with β2-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-β2-GPI antibody binding to membrane-bound β2-GPI, enabling domain I of β2-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 β2-GPI-dependent phosphatidylserine and cardiolipin ELISA in the presence of LMWH or unfractionated heparin.[5–7] Further, affinity chromatography with unfractionated heparin and LMWH columns adsorbed a significant proportion of aPL from sera of APS women with recurrent pregnancy loss.[6,7]

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.[39] Hydrogen bonds are also important, at least in some cases of protein–heparin interaction.[39] The interacting groups within heparin and proteins must be appropriately positioned and oriented to confer the specificity of heparin–protein interactions.[39] 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.[40] Further, arginine residues were implicated in binding of human monoclonal aPL derived from an APS patient to β2-GPI.[41] 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 β2-GPI.

An additional mechanism might exist through which nadroparin restores membrane adhesion in the presence of anti-β2-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 phosphatdylcholine from phospholipid vesicles.[42] This could further increase β2-GPI-induced adhesion between POPS-GPVs and could at least partly explain the significantly larger membrane adhesion in the presence of β2-GPI, anti-β2-GPI IgG, and nadroparin than in the presence of β2-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 β2-GPI-induced membrane adhesion in the presence of anti-β2-GPI antibodies suggests a direct role of anti-β2-GPI antibodies in enhancing membrane microvesiculation and is consistent with the finding that anti-β2-GPI antibodies directed against domain I strongly correlate with thrombosis in APS.[43] In the presence of anti-β2-GPI IgG antibodies, nadroparin completely restored the interactions between negatively charged phospholipid membranes and β2-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 β2-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.[44–47]

Acknowledgments

The work was supported by Ministry of High Education, Science and Technology of the Republic of Slovenia, number P3 0314 to B.R. The authors would like to thank Irena Jurgec, Dragica Petric, and Saša Čučnik for the

isolation and preparation of reagents; Aleš Iglič for constructive critique; and Rok Blagus for his valuable help with statistical analysis of data.

Conflicts of Interest

The authors declare no conflicts of interest.

References

1. Di Simone, N., M.P. Luigi, D. Marco, *et al*. 2007. Pregnancies complicated with antiphospholipid syndrome: the pathogenic mechanism of antiphospholipid antibodies. *Ann. N.Y. Acad. Sci.* **1108:** 505–514.
2. Empson, M., M. Lassere, J.C. Craig, *et al*. 2002. Recurrent pregnancy loss with antiphospholipid antibody: a systematic review of therapeutic trials. *Obstet. Gynecol.* **99:** 135–144.
3. Farquharson, R.G., S. Quenby & M. Greaves. 2002. Antiphospholipid syndrome in pregnancy: a randomized, controlled trial of treatment. *Obstet. Gynecol.* **100:** 408–413.
4. Guerin, J., Y. Sheng, S. Reddel, *et al*. 2002. Heparin inhibits the binding of β2-glycoprotein I to phospholipids and promotes the plasmin-mediated inactivation of this blood protein. Elucidation of the consequences of the two biological events in patients with the anti-phospholipid syndrome. *J. Biol. Chem.* **277:** 2644–2649.
5. Wagenknecht, D.R. & J.A. McIntyre. 1992. Interaction of heparin with β2-glycoprotein I and antiphospholipid antibodies in vitro. *Thromb. Res.* **68:** 495–500.
6. Franklin, R.D. & W.H. Kutteh. 2003. Effects of unfractionated and low molecular weight heparin on antiphospholipid antibody binding in vitro. *Obstet. Gynecol.* **101:** 455–462.
7. Ermel, L.D., P.B. Marshburn & W.H. Kutteh. 1995. Interaction of heparin with antiphospholipid antibody (APA) from the sera of women with reccurent pregnancy loss (RPL). *Am. J. Reprod. Immunol.* **33:** 14–20.
8. Masamoto, H., T. Toma, K. Sakumoto, *et al*. 2001. Clearance of antiphospholipid antibodies in pregnancies treated with heparin. *Obstet. Gynecol.* **97:** 394–398.
9. Di Simone, N., P.L. Meroni, M. D'Asta, *et al*. 2007. Pathogenic role of anti-β2-glycoprotein I antibodies on human placenta: functional effects related to implantation and roles of heparin. *Hum. Reprod. Update.* **13:** 189–196.
10. Dignat-George, F., L. Camoin-Jau, F. Sabatier, *et al*. 2004. Endothelial microparticles: a potential contribution to the thrombotic complications of the antiphospholipid syndrome. *Thromb. Haemost.* **91:** 667–673.
11. Distler, J.H., D.S. Pisetsky, L.C. Huber, *et al*. 2005. Microparticles as regulators of inflammation: novel players of cellular crosstalk in the rheumatic diseases. *Arthritis Rheum.* **52:** 3337–3348.
12. Jy, W., M. Tiede, C,J. Bidot, *et al*. 2007. Platelet activation rather than endothelial injury identifies risk of thrombosis in subjects positive for antiphospholipid antibodies. *Thromb. Res.* **121:** 319–325.
13. Combes, V., A.C. Simon, G.E. Grau, *et al*. 1999. In vitro generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant. *J. Clin. Invest.* **104:** 93–102.
14. Morel, O., L. Jesel, J.M. Freyssinet, *et al*. 2005. Elevated levels of procoagulant microparticles in a patient with myocardial infarction, antiphospholipid antibodies and multifocal cardiac thrombosis. *Thromb. J.* **3:** 15.
15. Carp, H., R. Dardik, A. Lubetsky, *et al*. 2004. Prevalence of circulating procoagulant microparticles in women with recurrent miscarriage: a case controlled study. *Hum. Reprod.* **19:** 191–195.
16. Germain, S.J., G.P. Sacks, S.R. Sooranna, *et al*. 2007. Systemic inflammatory priming in normal pregnancy and preeclampsia: the role of circulating syncytiotrophoblast microparticles. *J. Immunol.* **178:** 5949–5956.
17. Sugimura, M., T. Kobayashi, F. Shu, *et al*. 1999. Annexin V inhibits phosphatidylserine-induced intrauterine growth restriction in mice. *Placenta* **20:** 555–560.
18. Pierangeli, S.S., P.P. Chen & E.B. González. 2006. Antiphospholipid antibodies and the antiphospholipid syndrome: an update on treatment and pathogenic mechanisms. *Curr. Opin. Hematol.* **13:** 366–375.
19. Ambrožič, A., B. Božič, T. Kveder, *et al*. 2005. Budding, vesiculation and permeabilization of phospholipid membranes - evidence for a feasible physiologic role of β2-glycoprotein I and pathogenic actions of anti-β2-glycoprotein I antibodies. *Biochim. Biophys. Acta.* **1740:** 38–44.
20. Urbanija, J., N. Tomšič, M. Lokar, *et al*. 2007. Coalescence of phospholipid membranes as a possible origin of anticoagulant effect of serum proteins. *Chem. Phys. Lipids.* **150:** 49–57.
21. Bohinc, K., A. Iglič & S. May. 2004. Interaction between macroions mediated by divalent rod-like ions. *Europhys. Lett.* **68:** 494–500.
22. May, S., A. Iglič, J. Reščič, *et al*. 2008. Bridging like-charged macroions through long divalent rod-like ions. *J. Phys. Chem. B.* **112:** 1685–1692.
23. Urbanija, J., K. Bohinc, A. Bellen, *et al*. 2008. Attraction between negatively charged surfaces mediated by

spherical counterions with quadrupolar charge distribution. *J. Chem. Phys.* **129:** 105101.
24. Urbanija, J., B. Babnik, M. Frank, *et al*. 2008. Attachment of β2-glycoprotein I to negatively charged liposomes may prevent the release of daughter vesicles from the parent membrane. *Eur. Biophys. J.* **37:** 1085–1095.
25. Pavlič, J.I., T. Mareš, J. Bešter, *et al*. 2008. Encapsulation of small spherical liposome into larger flaccid liposome induced by human plasma proteins. *Comput. Methods Biomech. Biomed. Engin. Preprint*. [DOI: 10.1080/10255840802560326].
26. Frank, M., M. Manček-Keber, M. Kržan, *et al*. 2008. Prevention of microvesiculation by adhesion of buds to the mother cell membrane - a possible anticoagulant effect of healthy donor plasma. *Autoimmun. Rev.* **7:** 240–245.
27. Janša, R., V. Šuštar, M. Frank, *et al*. 2008. Number of microvesicles in peripheral blood and ability of plasma to induce adhesion between phospholipid membranes in 19 patients with gastrointestinal diseases. *Blood Cells Mol. Dis.* **41:** 124–132.
28. Angelova, M.I., S. Soléau, P. Méléard, *et al*. 1992. Preparation of giant vesicles by external AC electric fields. Kinetics and applications. *Progr. Colloid. Polym. Sci.* **89:** 127–131.
29. Brighton, T.A., P.J. Hogg, Y.P. Dai, *et al*. 1996. β2-glycoprotein I in thrombosis: evidence for a role as a natural anticoagulant. *Br. J. Haematol.* **93:** 185–194.
30. Čučnik, S., T. Kveder, I. Križaj, *et al*. 2004. High avidity anti-β2-glycoprotein I antibodies in patients with antiphospholipid syndrome. *Ann. Rheum. Dis.* **63:** 1478–1482.
31. Hashimoto, Y., M. Kawamura, K. Ichikawa, *et al*. 1992. Anticardiolipin antibodies in NZW x BXSB F1 mice. A model of antiphospholipid syndrome. *J. Immunol.* **149:** 1063–1068.
32. Ichikawa, K., A. Tsutsumi, T. Atsumi, *et al*. 1999. A chimeric antibody with the human gamma1 constant region as a putative standard for assays to detect IgG β2-glycoprotein I-dependent anticardiolipin and anti-β2-glycoprotein I antibodies. *Arthritis Rheum.* **42:** 2461–2470.
33. Menger, M.F. & M.I. Angelova. 1998. Giant vesicles: imitating the cytological processes of cell membranes. *Acc. Chem. Res.* **31:** 789–797.
34. Sodin-Šemrl, S., M. Frank, A. Ambrožič, *et al*. 2008. Interactions of phospholipid binding proteins with negatively charged membranes: β2-glycoprotein I as a model mechanism. In *Advances in Planar Lipid Bilayers and Liposomes*, Vol. 8. A. Leitmannova Liu, Ed.: 243–273. Elsevier Academic Press. Amsterdam, NL.
35. Dachary-Prigent, J., J.M. Pasquet, J.M. Freyssinet, *et al*. 1995. Calcium involvement in aminophospholipid exposure and microparticle formation during platelet activation: a study using Ca2+ -ATPase inhibitors. *Biochemistry* **34:** 11625–11634.
36. Lee, A.T., K. Balasubramanian & A.J. Schroit. 2000. β(2)-glycoprotein I-dependent alterations in membrane properties. *Biochim. Biophys. Acta.* **1509:** 475–484.
37. Hamdan, R., S.N. Maiti & A.J. Schroit. 2007. Interaction of β2-glycoprotein I with phosphatidylserine-containing membranes: ligand-dependent conformational alterations initiate bivalent binding. *Biochemistry* **46:** 10612–10620.
38. Polz, E., H. Wurm & G.M. Kostner. 1980. Investigations on β2-glycoprotein I in the rat: isolation from serum and demonstration in lipoprotein density fractions. *Int. J. Biochem.* **11:** 265–270.
39. Capila, I. & R.J. Linhardt. 2002. Heparin-protein interactions. *Angew. Chem. Int. Ed. Engl.* **41:** 390–412.
40. Giles, I.P., J.D. Haley, S. Nagl, *et al*. 2003. A systematic analysis of sequences of human antiphospholipid and anti-β2-glycoprotein I antibodies: the importance of somatic mutations and certain sequence motifs. *Semin. Arthritis Rheum.* **32:** 246–265.
41. Giles, I., N. Lambrianides, N. Pattni, *et al*. 2006. Arginine residues are important in determining the binding of human monoclonal antiphospholipid antibodies to clinically relevant antigens. *J. Immunol.* **177:** 1729–173.
42. Vannucchi, S., M. Ruggierro & V. Chiarugi. 1985. Complexing of heparin with phosphatidylcholine. A possible supramolecular assembly of plasma heparin. *Biochem. J.* **227:** 57–65.
43. de Laat, B., R.H. Derksen, R.T. Urbanus, *et al*. 2005. IgG antibodies that recognize epitope Gly40-Arg43 in domain I of β2-glycoprotein I cause LAC and their presence correlates strongly with thrombosis. *Blood* **105:** 1540–1545.
44. Sodin-Šemrl, S. & B. Rozman. 2007. β2-glycoprotein I and its clinical significance: from gene sequence to protein levels. *Autoimmun. Rev.* **6:** 547–552.
45. Abid Husein, M.N., A.N. Böing, E. Biró, *et al*. 2008. Phospholipid composition of in vitro endothelial microparticles and their in vivo thrombogenic properties. *Thromb. Res.* **121:** 865–871.
46. Koike, T., M. Bohgaki, O. Amengual, *et al*. 2007. Antiphospholipid antibodies: lessons from the bench. *J. Autoimmun.* **28:** 129–133.
47. Shoenfeld, Y., G. Twig, U. Katz, *et al*. 2008. Autoantibody explosion in antiphospholipid syndrome. *J. Autoimmun.* **30:** 74–83.