The Effect of *in vitro* Anticoagulant Disodium Citrate on Beta-2-glycoprotein I - Induced Coalescence of Giant Phospholipid Vesicles

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Abstract— **In order to elucidate the mechanisms of blood coagulation the complex interactions between phospholipid membranes, serum protein beta-2-glycoprotein I (β_2GPI), antiphospholipid antibodies (aPL) and disodium citrate were studied by observing collective interactions between giant phospholipid vesicles (GPVs) in the sugar solution. GPVs composed of palmitoyl-oleoyl-sn-glycero-3-phospho-choline (POPC), tetraoleoyl cardiolipin and cholesterol were obtained by the electroformation method and observed under the phase contrast microscope. β_2GPI or aPL acted as mediators inducing the coalescence of the vesicles. The strength of the adhesion between the coalesced vesicles was dependent on the content of cardiolipin and the species of the mediator. The addition of disodium citrate to the coalesced GPVs solution caused disintegration of the complexes of coalesced vesicles. The extend of the disintegration between coalesced vesicles was interpreted to be connected to the strength of the adhesion between GPVs. It was found that the disintegration of the GPV complexes was more pronounced in the system where the vesicles coalesced due to the presence of antiphospholipid antibodies compared to the system where the vesicles coalesced due to the presence of β_2GPI. The effect of the disintegration of the coalesced GPVs was more pronounced for smaller vesicles which originated in the budding of the the membrane of larger GPVs.**

Keywords— **Beta-2-glycoprotein I, Antiphospholipid antibodies, Giant phospholipid vesicles, Thrombosis.**

I. Introduction

Antiphospholipid syndrome (APS) is a complex clinical syndrome characterized by recurent vascular thrombosis, pregnancy morbidity and thrombocytopenia, which occur in the presence of antiphospholipid antibodies (aPL) (1). aPL are a heterogenous group of antibodies. Only aPL directed against phospholipid binding proteins were asociated with clinical manifestations of APS (2). Beta-2-glycoprotein I (β_2GPI), a phospholipid-binding plasma protein, is the major antigen for aPL (3). The role of β_2GPI and aPL in the blood coagulation is not well understood, however β_2GPI was found to be anticoagulant *in vivo* while aPL were found to be procoagulant *in vivo*. Sodium citrate is used to prevent the coalescence of blood cells *in vitro*. In order to elucidate the mechanisms of blood coagulation, it is of interest to better understand complex interactions between phospholipid membranes, procoagulants and anticoagulants *in vivo* as well as anticoagulants *in vitro*.

It was previously shown that β_2GPI, monoclonal antibodies against β_2GPI (maβ_2GPIs) and their combination bind to phospholipid surfaces and may induce the coalescence of giant phospholipid vesicles (GPVs) (4, 5).

In our present work we wanted to estimate the strength of the adhesive interactions between GPVs mediated by β_2GPI or maβ_2GPIs by adding disodium citrate to the solution with coalesced GPVs. Disodium citrate causes the disintegration of the complexes formed by the vesicles.

II. Materials and methods

A. β_2GPI *and Monoclonal Anti* β_2GPI *Antibodies*

β_2GPI and maβ_2GPIs antibodies (Hyphen BioMed, France) were aliquoted and stored at - 70°C. In all experiments, the final concentration of β_2GPI in phosphate buffer saline (PBS) was 200 mg/L, which is the concentration of physiological β_2GPI in normal human plasma (about 200 mg/L) (6,7). The final concentraction of maβ_2GPI dissolved in PBS used in experiments was 200 mg/ml.

B. *Disodium citrate solution*

Disodium citrate was dissolved in water at the concentration of 200 mmol/L and further diluted for the experiments. In all experiments, the final concentrations of disodium citrate was 0,046 mol/l.

C. *Giant phospholipid vesicles*

GPVs were prepared at room temperature (23°C) by the modified electroformation method (8). The synthetic lipids cardiolipin (1,1'2,2'-tetraoleoyl cardiolipin), POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), and cho-

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lesterol were purchased from Avanti Polar Lipids, Inc. Appropriate volumes of POPC, cardiolipin and cholesterol, all dissolved in a 2:1 chloroform/methanol mixture, were combined in a glass jar and thoroughly mixed. For vesicles containing 20% and 40% weight ratio of negatively charged cardiolipin POPC, cholesterol and cardiolipin were mixed in the proportions 3:1:1 and 2:1:2 respectively. 20 μL of lipid mixture was applied to the platinum electrodes. The solvent was allowed to evaporate in a low vacuum for 2 hours. The coated electrodes were placed in the electroformation chamber which was then filled with 3 mL of 0.2 M sucrose solution. An AC electric current with an amplitude of 5 V and a frequency of 10 Hz was applied t

o the electrodes for 2 hours, which was followed by 2.5 V and 5 Hz for 15 minutes, 2.5 V and 2.5 Hz for 15 minutes and finally 1 V and 1 Hz for 15 minutes. The content was rinsed out of the electroformation chamber with 5 mL of 0.2 M glucose and stored in a plastic test tube at 4°C. The vesicles were left for sedimentation under gravity for one day and were then used for a series of experiments.

D. Observation

The vesicles were observed by an inverted microscope Zeiss Axiovert 200 with phase contrast optics and recorded by the Sony XC-77CE video camera. The solution containing vesicles was placed into the observation chamber made from cover glasses sealed with grease. The larger (bottom) cover glass was covered by two smaller cover glasses, each having a small semicircular part removed at one side. Covering the bottom glass by two opposing cover glasses formed a circular hole in the middle of the observation chamber. In all experiments the solution of vesicles (45 μl) was placed in the observation chamber. The solution containing the substance under investigation (β_2GPI or maβ_2GPI, followed by disodium citrate) was added into circular opening in the middle of the observation chamber.

5 μl of disodium citrate solution was repeatedly added to the solution containing coalesced GPVs 30, 45 and 60 minutes following the addition of either β_2GPI or maβ_2GPI. Together, 15 μl of disodium citrate solution was added to the solution of coalesced GPVs.

III. Results

A. Addition of either b2GPI or maB2GPI to the giant phospholipid vesicles

The solution of GPVs contained a heterogenous population of vesicle shapes with many vesicles exhibiting tubular protrusions (Fig. 1). Most vesicles were flaccid and were fluctuating thermally. Few minutes after the addition of either β_2GPI or maβ_2GPI to the GPVs solution the thermal fluctuations of vesicles diminished, the tubular protrusions disintegrated into smaller vesicles and the GPVs became nearly spherical (Fig. 2). The observed shape transformation was attributed to the effect of the PBS since the same effect was observed also after the addition of PBS alone. With PBS alone the sample ultimately contained nearly spherical fluctuating vesicles.

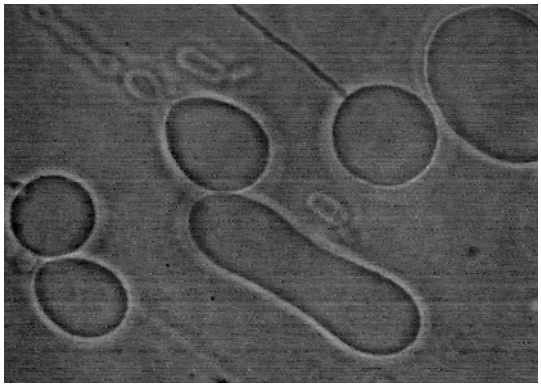

Fig. 1: The solution of giant phospholipid vesicles containing 40% weight ratio of cardiolipin.

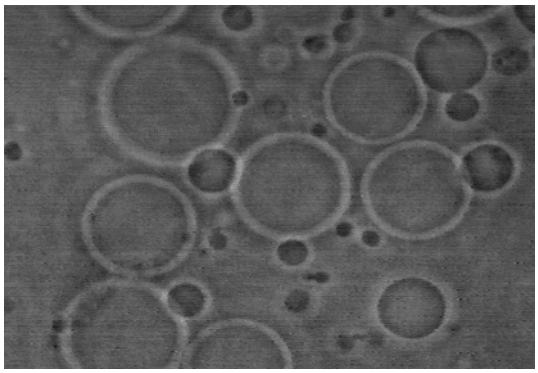

Fig. 2: The solution of giant phospholipid vesicles containing 40% weight ratio of cardiolipin few minutes after the addition of monoclonal antibodies against β_2GPI dissolved in PBS to the solution with vesicles.

B. Addition of disodium citrate solution to the solution with coalesced GPVs

β_2GPI as well as maβ_2GPI caused the vesicles to coalesce into two- or multicompartment complexes (Figs. 3 and 4) which adhered to the bottom of the glass slide and ceased to fluctuate. The adhesion of the vesicles was stronger in the GPV-β_2GPI solution compared to the GPV-maβ_2GPI solution as the areas of contact between adhered

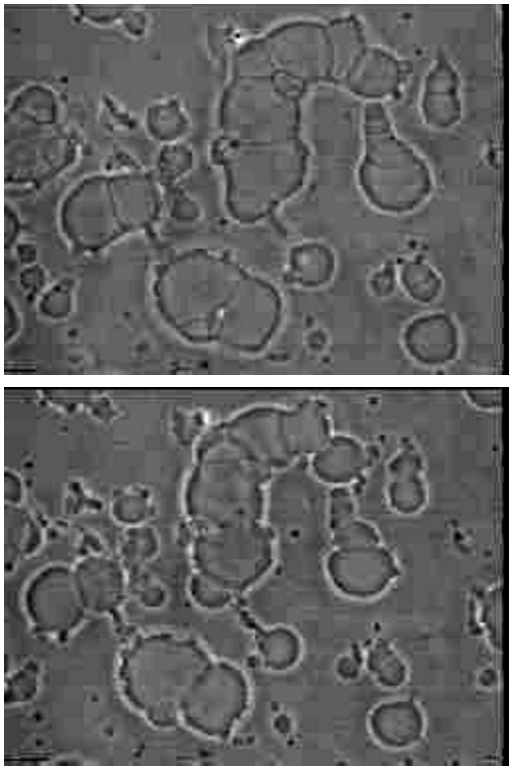

Fig. 3: The adhesion of giant phospholipid vesicles containing 40% weight ratio of cardiolipin due to the addition of β_2GPI (upper). The addition of 15 μl disodium citrate caused only slight disintegration of the complexes (lower).

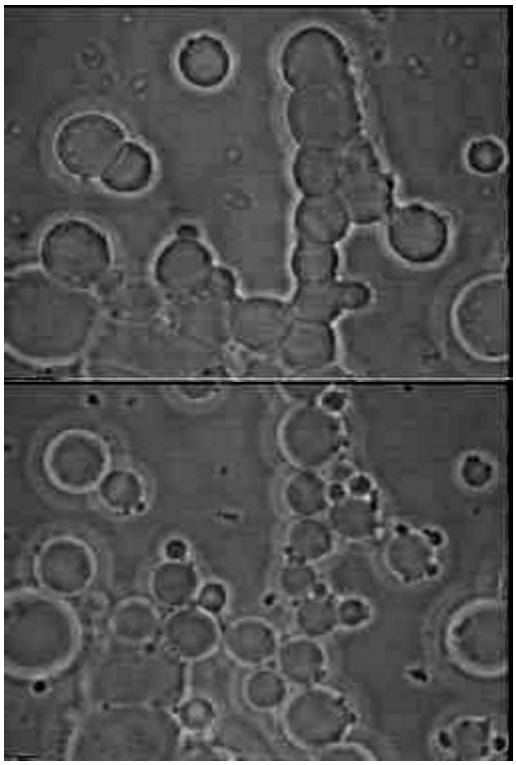

Fig. 4. The adhesion of giant phospholipid vesicles containing 40% weight ratio of cardiolipin due to the addition of monoclonal antibodies against β_2GPI (upper). Disodium citrate caused substantial disintegration of th complexes (lower).

vesicles seemed larger in the GPV-β_2GPI solution compared to the GPV-maβ_2GPI solution. Slight disintegration of the coalesced GPVs was observed in the GPV-β_2GPI solution about 5 minutes after the addition of disodium citrate (Fig. 4) while the effect was pronounced in the GPV-maβ_2GPI solution. The disintegration was most prominent for smaller vesicles, which originated in the budding from the membranes of large GPVs (Fig.4) while in GPV-β_2GPI solution, the GPV complexes were preserved even after the third addition of 5 μl of disodium citrate (Fig. 3).

It was also found that the adhesive interactions between GPVs are stronger for higher cardiolipin content (not shown).

IV. Discussion

GPVs are a convenient *in vitro* system for studying the interactions between phospholipid membranes and proteins. The observation of membrane adhesions between GPVs could contribute to the better understanding of the role of plasma proteins and anticoagulant drugs in thrombosis and haemostasis.

The mechanisms causing the adhesion between phospholipid membranes may be of different origins. In β_2GPI, a domain carrying a net positive charge and a domain that can by virtue of hydrophobic interactions insert into the phospholipid layer were identified. Such configuration of the β_2GPI molecule can be involved in a bridging mechanism thereby establishing attractive interaction between like-charged membranes in close contact. In the case of antibodies, dimeric structure of antibodies can give rise to spatially distributed charge that can be described as a quadrupole. Quadrupolar ordering of the antibodies in the gradient of the electric field of the membrane may lower the free energy of the system. Further, this particular configuration of charge can be effectively described as direct interaction between charges in the electric double layer model which yields an attractive interaction between the like-charged membranes.

We have shown that there is a difference in the disodium citrate effects in the solutions of coalesced vesicles with different interaction mediators. However, the mechanism underlying the effect of the disodium citrate on the coalesced complexes remains obscure.

V. Conclusions

Disodium citrate caused disintegration of coalesced GPV complexes. The effect was stronger if the contact between the vesicles and between the ground support was weaker.

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