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Interaction of giant phospholipid vesicles containing cardiolipin and cholesterol with β2-glycoprotein-I and anti-β2-glycoprotein-I antibodies

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Abstract

Antiphospholipid syndrome is characterized with thrombotic events and/or pregnancy morbidity and antiphospholipid antibodies (aPL). The most common antigen for aPL is beta2-glycoprotein-I (β_2GPI), a plasma protein binding to negatively charged phospholipids. The influence of aPL on coagulation is not well understood. Giant phospholipid vesicles (GPVs) are a convenient in vitro system for studying interactions between phospholipid membranes and proteins resulting in the change of the vesicles' configuration. We aimed to set up an in vitro model and to study changes in the morphology of GPVs with high content of cardiolipin upon addition of β_2GPI and/or IgG fraction of a patient with antiphospholipid syndrome (APS). Addition of the IgG fraction of the APS patient caused lateral segregation of the membrane inclusions and adhesion of GPVs. Addition of β_2GPI caused adhesion of GPVs. Addition of both, the patient IgG fraction and β_2GPI caused adhesion of vesicles to the glass slides and to each other, formation of pores and burst of vesicles. Our results indicate that adhesion of the cardiolipin-containing vesicles does not seem specific for added proteins, rather, it indicates electrostatic and curvature-mediated interactions between the membrane constituents.
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Keywords: Giant phospholipid vesicles; β2-glycoprotein-I; Anti-β2-glycoprotein-I antibodies

Contents

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1. Introduction

Antiphospholipid syndrome (APS) is a complex clinical syndrome characterized by thrombotic events and/or pregnancy morbidity and the presence of antiphospholipid antibodies (aPL) [1]. aPL is a heterogeneous group of antibodies. Only aPL directed against phospholipid binding proteins were associated with clinical manifestations of APS [2]. The most common antigen for aPL is beta2-glycoprotein-I (β_2GPI) [3]. Interactions between phospholipids, β_2GPI and antibodies against β_2GPI (anti-β_2GPI) are of great interest in pursuing the understanding of mechanisms of pathogenic action of aPL. These mechanisms have been partially revealed, however, the understanding of the influence of aPL on coagulation is yet fragmentary. Additionally, the physiological role of β_2GPI is not yet completely understood and valuable information may be obtained from in vitro studies.

Giant phospholipid vesicles (GPVs) are convenient in vitro system for studying interactions between phospholipid membranes and protein cofactors as they could be detected by the change of the vesicles' configuration (shape and lateral distribution of their constituents). It was previously found that a solution containing β_2GPI, induced budding and consequent fragmentation of the membrane in giant phospholipid vesicles containing 10% weight ratio of phosphatidylserine [4]. The solution containing an IgG fraction of a patient with APS did not cause vesicle shape change, however when β_2GPI was added to the IgG fraction in the solution the effect of budding and fragmentation was more pronounced than with β_2GPI alone [4]. Cardiolipin containing GPVs seems the most relevant model for studying β_2GPI binding and the role of anti-β_2GPI antibodies.

The aim of this work was to establish an in vitro model to study the influence of β_2GPI and an IgG fraction of a patient with APS on GPVs with high weight fraction of cardiolipin [5]. Our in vitro model enabled us to observe morphological changes of GPVs after addition of β_2GPI, the IgG fraction of a patient with APS and both fractions, to the solution that contained vesicles.

2. Materials and methods

2.1. β_2GPI and anti-β_2GPI antibodies

β_2GPI (Hyphen BioMed, France) was aliquoted and stored at −70 °C. In all experiments, the final concentration of β_2GPI was 100 mg/L, which is approximately half the concentration of physiological β_2GPI in normal human plasma (about 200 mg/L) [6,7].

IgG fractions were isolated from the serum of a patient with primary APS containing high titers of IgG anti-β_2GPI by affinity purification on a protein-G column (Pierce, Rockford, USA), following manufacturer's recommendations. IgG fractions were equilibrated against phosphate buffer saline (PBS, pH 7.4) in a desalting column. The IgG fraction of a patient with primary APS gave comparable results as the whole serum in the anti-β_2GPI ELISA performed previously [8]. However, in a modified aCL ELISA [9] this IgG fraction and serum showed significant background binding indicating the presence of aPL binding directly to cardiolipin in the absence of β_2GPI or any other cofactor.

2.2. Giant phospholipid vesicles

GPVs were prepared at room temperature (23 °C) by the modified electroformation method [10]. Synthetic lipids: cardiolipin (1,1′2,2′-Tetraoleoyl Cardiolipin), POPC (1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine), and cholesterol were purchased from Avanti Polar Lipids, Inc. Adequate volumes of POPC, cardiolipin and cholesterol, all dissolved in 2:1 chloroform/methanol mixture, were combined in a glass jar and thoroughly mixed. Then, 20 μl of the mixture was applied to platinum electrodes. The solvent was allowed to evaporate in low vacuum for 2 h. The coated electrodes were placed in the electroformation chamber and 3 ml of 0.2 M sucrose solution was poured into the chamber. An AC electric current with amplitude 5 V and frequency 10 Hz was applied to the electrodes for 2 h, which was followed by 2.5 V and 5 Hz for 15 min, 2.5 V and 2.5 Hz for 15 min and finally 1 V and 1 Hz for 15 min. The content was rinsed

out of the electroformation chamber with 5 ml of 0.2 M glucose solution and used immediately after the formation or stored at 4 °C. Vesicles were observed by an inverted microscope Zeiss Axiovert 200 with phase contrast optics and recorded by the Sony XC-77CE video camera. We used vesicles that were not older than one week as the samples eventually became contaminated by bacteria.

2.3. Observation

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 (18 × 18 mm glasses), each having a small semicircular part removed at one side. Covering the

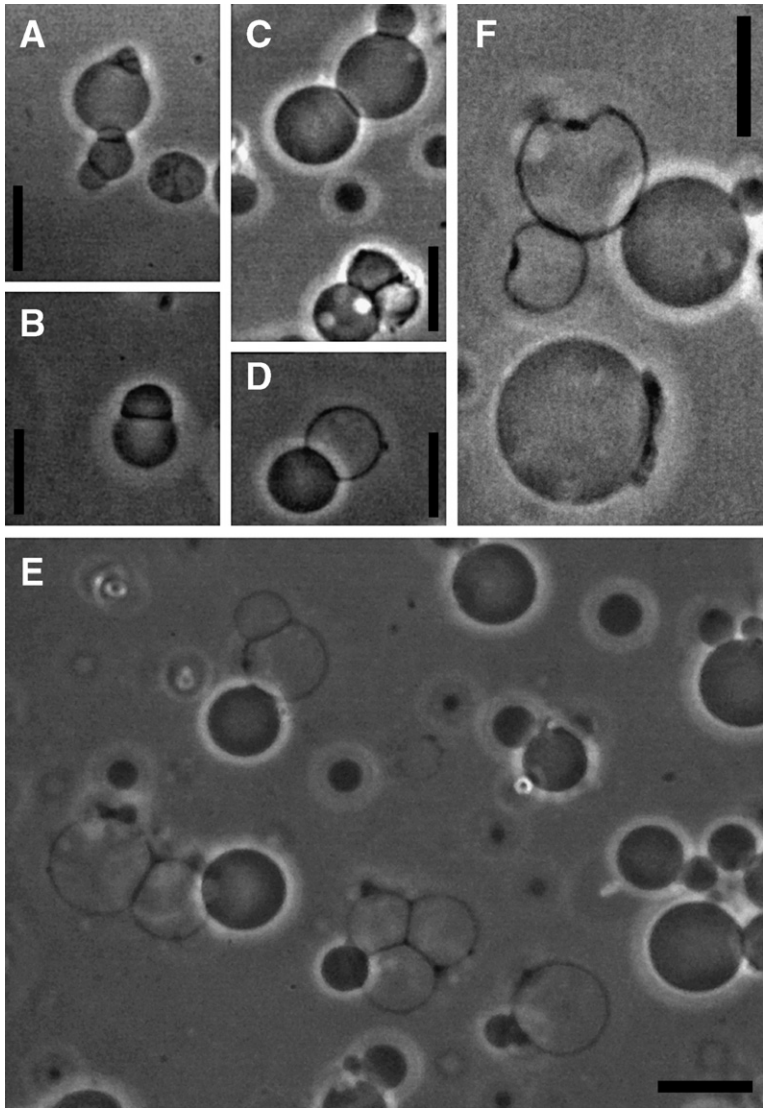

Fig. 1. (A–D): Effect of β_2GPI and IgG fraction of a patient with antiphospholipid syndrome on GUVs containing cardiolipin and cholesterol. Sticky formations induced by addition of β_2GPI (A and B) and by addition of IgG fraction of a patient with antiphospholipid syndrome (C and D). A flat wall dividing the vesicular compartments (area of contact of the two vesicles) is indicated as the two solutions are of different contents (D). The areas of contact seem larger after addition of β_2GPI (A and B). Regions of the lateral separation can be observed after the addition of IgG fraction of a patient with antiphospholipid syndrome (C and D). (E) The sample with cardolipin-containing vesicles. IgG fraction of a patient with antiphospholipid syndrome was added to the solution containing the vesicles, causing the vesicles to adhere to the bottom of the glass slide and to each other. After about 1 h, β_2GPI, dissolved in PBS, was added to the solution. Many vesicles transformed into ghosts indicating formation of membrane pores. (F) cardiolipin-containing vesicles after addition of IgG fraction of a patient with antiphospholipid syndrome. Lateral separation within the membrane is indicated (darker regions within the membrane). Some vesicles transformed into ghosts (light vesicles) indicating formation of membrane pores. Bars denote 10 μm.

bottom glass by two opposing smaller 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 (5 μl) was added into the circular opening in the middle of the observation chamber. This was performed very gently, by negligibly disturbing the sample, so that the same vesicles could be observed before and after the addition of the dissolved proteins.

The osmolarity of the sample containing vesicles (measured by Knauf Semiosmometer) was 205 mosm/L, while PBS had an osmolarity of 280 mosm/L. We assumed that the presence of proteins dissolved in PBS caused only a negligible difference.

3. Results

3.1. A) Addition of β_2GPI to giant phospholipid vesicles

The solution of GPVs contained a heterogeneous population of shapes. Many vesicles exhibited tubular protrusions. Most of the vesicles were flaccid. Thermal fluctuations of shapes were notable. Few minutes after the addition of the solution containing dissolved β_2GPI, thermal fluctuations of vesicles diminished, protrusions disintegrated into spherical fragments while vesicles attained nearly spherical shapes. This phase of the morphological changes was observed also after addition of PBS alone. With PBS, the sample ultimately contained nearly spherical fluctuating vesicles. With β_2GPI however, nearly spherical fragments joined into two or multi-compartment structures composed of spherical parts and flat walls (Fig. 1A,B). Furthermore, vesicles adhered to the bottom of the glass slide and ceased to fluctuate. Large surfaces of contact between spherical compartments could be observed. The sample ultimately contained motionless aggregates of vesicles adhered to the bottom of the glass slide. Vesicles in the sample retained their shape until the end of observation (one hour).

3.2. B) Addition of an IgG fraction isolated from a patient with APS to giant phospholipid vesicles

When the IgG fraction of a patient with APS was added to the solution with GPVs a similar time course was observed as when β_2GPI was added to GPVs (Fig. 1C,D). However, in addition to the suppression of fluctuations and adhesion of vesicles, we observed lateral segregation within the membrane in the time scale of minutes (Fig. 1F). The contour of the vesicles became irregular in thickness (Fig. 1F). Eventually, regions of higher local curvature could be observed and the vesicles became deformed from their shapes composed of spherical segments (Fig. 1F). Ghosts (light vesicles, indicating that the membrane became permeable to sugar) were found within the population (Fig. 1D,E,F). GPVs became motionless and adhered to the bottom of the glass slide about 30 min following addition of the IgG fraction.

We observed a difference between the sample with the IgG fraction of a patient with APS and the sample with β_2GPI. The adhesion of vesicles seemed less strong in the system with the IgG fraction than in the system with β_2GPI (compare Fig. 1A, B and C, D). The areas of contact seemed larger in the system with β_2GPI than in the system with IgG fraction.

3.3. C) Addition of both β_2GPI and the IgG fraction of a patient with APS to giant phospholipid vesicles

In the next step we added β_2GPI to the solution of GPVs one hour following addition of the IgG fraction. We have observed additional transformations of vesicles into ghosts and also sudden bursts of some vesicles. The sample was eventually formed from adhered vesicles and ghosts and bi- and multi-compartment vesicle and ghost formations, some exhibiting lateral segregation and regions of larger curvature (Fig. 1E). The reversed order of addition of β_2GPI and IgG fraction in the same time intervals resulted in similar observations, however, we did not directly observe bursts of the membrane.

4. Discussion

Negative charge on phospholipid membranes is expected to be important for the interaction between membrane and protein fractions. β_2GPI interacts with negatively charged phospholipid membranes with a patch of positively charged amino-acid residues and a hydrophobic loop on the fifth domain of β_2GPI [11]. In the present study we investigated interactions of β_2GPI and anti-β_2GPI with highly charged cardiolipin containing membrane. For the purpose of this work we intended to compose vesicles with a high content of cardiolipin as possible due to known interactions of β_2GPI with negatively charged phospholipids. Therefore we composed GPVs from one-third weight fraction of POPC, one-third weight fraction of cardiolipin and one-third weight fraction of cholesterol. Samples were rich in vesicles. They proved to be stable for many days and generally exhibited no phase separation. Thin tubes

attached to the globular part were observed, similar as in the pure POPC vesicles where they were recognized as a reservoir for the membrane area in the shape transformations of the vesicles [12].

Adding PBS to the suspension of vesicles caused evagination, i.e. an increase of the average mean curvature of the vesicles. This can be explained by the difference in the osmolarity of the outer solution of vesicles (205 mosm) and the added PBS (280 mosm). Water migration from the vesicles to the outer solution decreased the volume-to-area ratio, which rendered the vesicles flaccid and enabled evagination of the membrane.

In our experiments we have additionally observed ghosts, i.e. the membrane became permeable to sugar while retaining its integrity suggesting that pores may be formed in the membrane. The embedded protein molecules may form together with the lipid molecule inclusions in which non-lamellar assembly of cardiolipin is favored. This is in agreement with previous reports that cardiolipin may favor a non-lamellar configuration, which can be explained by its highly anisotropic structure [13].

Comparing the results of this work with previous results [4] we found that the previously observed effect of permeabilisation of GPVs in the presence of β_2GPI and anti-β_2GPI was confirmed also in this study. There are however differences that reveal new interesting features. In the previous study [4], a single vesicle was observed after transfer by a micropipette into the solution. The concentrations within the microenvironment of the vesicle were very well defined and controlled. In the present work, we could observe the collective effects of multiple vesicles which was previously not possible. Additionally, the current method is simple to perform in comparison to the micropipette transfer technique and reproducible. Most important, in previous experiments [4], the IgG fraction of a patient with APS alone seemed to have no effect on the single vesicle, while in this study, important effects were observed. This can be explained by different phospholipids and their concentrations used in the experiments. Previously [4] we used GPVs containing 10% phosphatidylserine, on the other hand, in the present study we used GPVs with 33% cardiolipin and 33% of cholesterol. The IgG preparation from patient with APS used in this work also contained the antibodies binding directly to cardiolipin that could cause some effects observed [4]. The rolleau formations have been reported to be formed by erythrocytes and were recognized as obstacles in blood stream [14].

The adhesion of vesicles and formation of multivesicular sticky structures observed in the presence of β_2GPI and IgG fraction of a patient with APS may represent processes that take place in the pathophysiologically important pathways leading to coagulation.

Take-home messages

- Giant phospholipid vesicles (GPVs) are convenient in vitro system for studying interactions between phospholipid membranes and proteins as they could be detected by the changes of the vesicles' configuration.
- Different synthetic lipids can be used for preparing GPVs including cardiolipin which seem the most relevant negatively charged phospholipid for studying β_2GPI binding and the role of anti-β_2GPI antibodies.
- Different techniques presented for GPVs observations gave similar results regarding permeabilisation of phospholipid membrane.
- The GPVs observations of lipid microparticles and membrane adhesion could play a role in procoagulant action of anti-β_2GPI antibodies.
- The current method enables a study of collective effects of multiple vesicles, which was previously not possible.

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Increased mesangial cell hyaluronan expression in lupus nephritis is mediated by anti-DNA antibody-induced IL-1beta

The mechanism by which anti-DNA antibodies contribute to the pathogenesis of lupus nephritis (LN) remains to be fully elucidated. Hyaluronan (HA) is an important extracellular matrix constituent that participates in lymphocyte recruitment to sites of inflammation. In this study, Yung S. et. al. (Kidney Int. 2006; 69: 272-80) investigated the expression of HA in renal biopsies and circulating HA levels in patients with diffuse proliferative LN, and the effect of human anti-DNA antibodies on HA synthesis in cultured human mesangial cells (HMC). HA expression was increased in the mesangium, and in the periglomerular and tubular distribution in LN kidney biopsies. LN patients showed increased levels of circulating HA, especially during active disease. Anti-DNA antibodies isolated during active LN but not remission increased de novo synthesis of (3)H-labeled HA, which was accompanied by induction of HA synthase (HAS) II transcription, and enhanced IL-1beta, IL-6, and tumor necrosis factor-alpha secretion in HMC ($p<0.001$ for all). These findings demonstrate that expression is significantly increased within the mesangium in diffuse proliferative LN mediated through anti-DNA antibody-induced IL-1beta. Given that HA plays a pivotal role during inflammatory responses, influences cellular behavior and assists in the recruitment of lymphocytes to sites of injury, it is likely that HA contributes to the pathogenesis of LN.

Differential binding of cross-reactive anti-DNA antibodies to mesangial cells: the role of alpha-actinin

Target Ag display is a necessary requirement for the expression of certain immune-mediated kidney diseases. Previous studies had shown that anti-DNA Abs that cross-react with alpha-actinin may be important in the pathogenesis of murine and human lupus nephritis. Furthermore, a pathogenic ant-DNA/alpha-actinin Ab showed enhanced binding to immortalized mesangial cells (MCs) derived from a lupus prone MRL-lpr/lpr mouse, suggesting that kidney alpha-actinin expression may be contributing to nephritis. In the current study, Zhao Z. et. al. (J Immunol 2006; 176: 7704-14) established that two isoforms of alpha-actinin that are present in the kidney, alpha-actinin 1 andalpha-actinin 4, can both be targeted by anti-alpha-actinin Abs. The authors found novel sequence polymorphisms between MRL-lpr/lpr and BALB/c in the gene for alpha-actinin 4. Moreover, alpha-actinin 4 and a splice variant of alpha-actinin 1 were both expressed at significantly higher levels (mRNA and protein) in MCs from the lupus prone MRL-lpr/lpr strain. It was concluded that enhanced alpha-actinin expression may determine the extent of Ig deposition in the Ab-mediated kidney disease in lupus. Modulation of Ag expression may be a promising approach to down-regulate immune complex formation in the target organ in individuals with circulating pathogenic Abs.