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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

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Abstract

The in vivo physiologic role of β2-glycoprotein I (β_2GPI) is presumed to be related to its interactions with negatively charged phospholipid membranes. Increased quantities of procoagulant microparticles derived by the vesiculation of blood cells have been detected in patients with antiphospholipid syndrome (APS) frequently associated with antibodies against β_2GPI (anti-β_2GPI). We investigated the influence of β_2GPI and anti-β_2GPI on giant phospholipid vesicles (GPVs). GPVs composed of phosphatidylserine and phosphatidylcholine were formed in an aqueous medium and individually transferred to a compartment containing either β_2GPI, anti-β_2GPI, or β_2GPI along with anti-β_2GPI. Shape changes of a single GPV were observed by a phase contrast microscope. Most GPVs transferred to the solution containing only β_2GPI budded moderately. Upon the transfer of GPVs to the solution containing β_2GPI and anti-β_2GPI either from patient with APS or mouse monoclonal anti-β_2GPI Cof-22, the budding was much more pronounced, generating also daughter vesicles. No such effects were seen when GPV was transferred to the solution containing anti-β_2GPI without β_2GPI. Our results suggest a significant physiologic role of β_2GPI in the budding of phospholipid membranes, which may be explained by the insertion of the C-terminal loop of β_2GPI into membranes, thus increasing the surface of the outer layer of a phospholipid bilayer. Anti-β_2GPI, recognizing domains I to IV of β_2GPI, enhanced the budding and vesiculation of GPVs in the presence of β_2GPI. This might be a novel pathogenic mechanism of anti-β_2GPI, promoting in vivo the expression of proadhesive and procoagulant phospholipid surfaces in APS.

Keywords: β2-glycoprotein I; Anti-β2-glycoprotein I; Antiphospholipid syndrome; Phospholipid membrane; Giant phospholipid vesicle

1. Introduction

β2-glycoprotein I (β_2GPI) is a 50 kDa plasma glycoprotein, which interacts with negatively charged phospholipids and has been recognized as the major antigen for antiphospholipid antibodies (aPL) [1]. β_2GPI consists of a single-chain (326 amino acids) folded into 5 homologous short consensus repeat domains [2,3]. The domain V of β_2GPI differs from the other four domains by two additional cysteines, responsible for the second internal loop and the long C-terminal tail [4]. Domain V also possesses a highly positively charged amino acid sequence Cys281–Cys288, which was shown to be a potent phospholipid binding site [5]. Recent X-ray analysis of β_2GPI showed that a patch consisting of 14 positively charged amino acid residues on domain V, as well as the flexible and partially hydrophobic C-terminal loop between Ser-311 and Lys-317, is involved in the binding to

Abbreviations: β_2GPI, β2-glycoprotein I; aPL, antiphospholipid antibodies; APS, antiphospholipid syndrome; anti-β_2GPI, antibodies against β_2GPI; GPV, giant phospholipid vesicle; POPS, 1-palmitoyl-2-oleoyl-phosphatidylserine; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; BSA, bovine serum albumin; PBS, phosphate buffer saline; AD, atopic dermatitis

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phospholipids [6]. The C-terminal loop contains an amino acid structure commonly seen in phospholipid binding proteins and it inserts into the outer layer of the phospholipid bilayer of biological membranes [6].

In clinical studies, antibodies against β_2GPI (anti-β_2GPI) were shown to be significantly associated with the signs of antiphospholipid syndrome (APS) [7,8]. Additionally, anti-β_2GPI were shown to be pathogenic, inducing thromboses and fetal loss in animal models [9–11]. In vitro studies have revealed some of the possible pathogenic actions of anti-β_2GPI, however, their entire pathogenic role is not fully understood yet (reviewed in Ref. [12]). Microparticles derived by the vesiculation of endothelial cells, platelets and other blood cells have been detected in normal human blood and in increased quantities in patients with APS [13,14]. aPL may also have the potential to permeabilize the synaptosomes, resulting in impaired neuronal functions, but the mechanism of this pathogenic action still remains unknown [15,16].

In biological research, there is a continuing search for appropriate models of biological cells in order to study certain elementary processes of a living cell in vitro. A simple model of the lipid matrix and aqueous interior are lipid vesicles (liposomes) with a diameter of 0.05 to a few μm. While liposomes can readily be prepared, they can be observed only by electron microscopy. On the other hand, giant vesicles are lipid vesicles with a diameter of 5 to 100 μm and can be directly studied by light microscopy in real time and video-recorded for further analyzes. The micropipette manipulation technique enables the observation of a single giant vesicle after it is added into a solution containing different reagents [17].

The interactions of β_2GPI and anti-β_2GPI with phospholipid membranes have already been studied by models employing liposomes [18–20], however this is the first report on interactions of β_2GPI and anti-β_2GPI with phospholipid membranes employing giant phospholipid vesicles (GPVs) as the simplest model of a cell membrane.

2. Materials and methods

2.1. Preparation of giant phospholipid vesicles (GPVs)

Unilamellar giant vesicles were prepared out of 10 mol% 1-palmitoyl-2-oleoyl-phosphatidylserine (POPS) and 90 mol% 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) lipid mixture (both from Avanti Polar-Lipids, Alabaster, AL, USA) according to a modified method of Angelova et al. [21]. The lipids, dissolved in chloroform–methanol, were spread over platinum electrodes and vacuum-dried for 2 h. The electrodes were placed in an electroformation chamber, filled with 2 ml of 0.2 M sucrose solution intended to be encapsulated in the vesicles. An AC field (1 V/mm, 10 Hz) was applied for 2 h and afterwards consecutively reduced in two 15-min steps (0.75 V/mm, 5 Hz; 0.5 V/mm, 2 Hz) to a final 30-min application of 0.25 V/mm and 2 Hz AC field. After the final step, the sample containing GPVs of different sizes (ranging from few micrometers to more than 100 μm) was washed out of the electroformation cell with an additional 2 ml of 0.2 M glucose solution and stored in a plastic beaker. Before the employment of GPVs, bovine serum albumin (BSA; essentially fatty acid free, Cat. no. A-6003, Sigma Chemical Company, St. Louis, MO, USA) in phosphate buffer saline (PBS), pH 7.4, was added. We chose PBS buffer and BSA concentrations that were shown previously to permit optimal interactions between β_2GPI and negatively charged phospholipid membranes [20]. The final preparation of GPVs contained 0.09 M sucrose, 0.09 M glucose, 0.015 M PBS and 0.5 mg/ml of BSA.

2.2. Micropipette transfer technique and observation of GPVs

An individual GPV of medium size (diameter about 40 to 60 μm) and without visible irregularities on the surface was aspirated by a glass micropipette from the compartment containing GPVs, and then transferred to the compartment containing β_2GPI (100 μg/ml) and/or anti-β_2GPI or purified normal human IgG in the control experiments. The diameter of the pipette was slightly larger than the diameter of the transferred vesicle. The time was measured from the moment when a vesicle was ejected from the micropipette to the test solution. The shape changes of a GPV, and in particular the formation of vesicle buds, were observed by a phase contrast microscope. In some experiments with Cof-22, this compound was added to the test solution at later times, dissolved in a medium that was identical to the test solution.

For the phase contrast microscopy, the Zeiss IM35 inverted microscope (objective Ph2, 40×, NA=0.65) was used. It was equipped with a black and white CCD camera (Sony SSC-M370CE) and a video recorder (Panasonic SVHS, AG-7350) for the image acquisition. Images were digitized on an 8-bit frame grabber (DT 2851, data Translation Inc) and analyzed with a homemade image software.

Under the phase contrast microscope, vesicles containing sucrose and floating in the glucose/sucrose solution appeared dark due to the difference in refraction indices of the sucrose and the glucose/sucrose solutions. There was another feature of the phase contrast: The rim around the object appeared with the opposite brightness than the object itself (halo). If the thickness of the object is larger than the depth of the focusing, the halo intensity depends only on the difference of the refraction indices for a given microscope [17]. The buds which were in the focus appeared darker than other vesicle parts, and the buds which were out of focus appeared brighter. The extent of budding was determined by counting the vesicles, which expressed buds in one experiment. In one experiment, we performed usually 10 transfers of vesicles. Each experiment was repeated 5 times, every

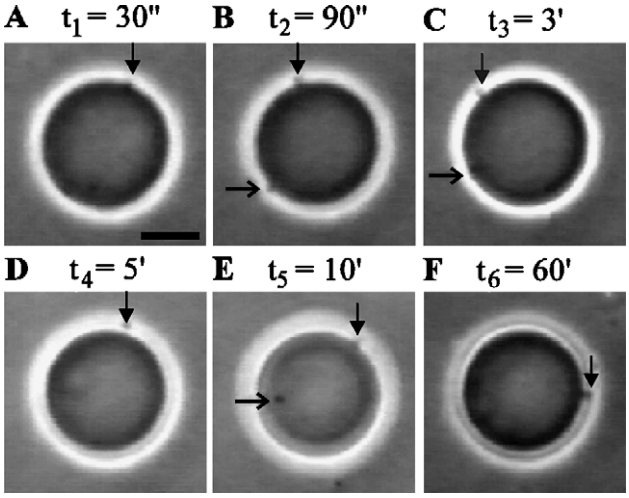

Fig. 1. Budding of GPVs in solution containing β_2GPI. (A, B) Two buds (arrows) were formed on the GPV, 90 s after the transfer of the GPV into the solution containing β_2GPI (the bar in Panel A indicates 20 μm). (C–F) No significant changes were seen during further observation for up to 60 min. On Panel D only one bud is visible, because the other one was not in the focus. (*t*: time in seconds (″) or minutes (′) after transferring GPV into the test solution).

time with different batch of vesicles. The operator of the microscope was not blind to the experiments, therefore to confirm his observations, the video recordings of the experiments were re-viewed by another expert. All experiments were conducted at room temperature (20 to 22 °C) to exclude thermally induced budding of phospholipid vesicles. This temperature was high above the gel to liquid crystal phase transition temperature, which is around −5 °C for our mixture of phospholipids.

2.3. β_2GPI and anti-β_2GPI antibodies

β_2GPI was purified from pooled human sera by affinity column chromatography [22], aliquoted and stored at −70 °C. In all experiments, the final concentration of β_2GPI was 100 μg/ml. The average plasma concentration of β_2GPI is about 200 μg/ml [23] and about 30% of the total plasma β_2GPI is lipid bound [24]. The concentration of β_2GPI used in our experiments was, therefore, close to the concentration of free β_2GPI in normal human plasma.

The monoclonal anti-β_2GPI Cof-22, obtained from BALB/c mice immunized with human β_2GPI [25], and recognizing domain III of β_2GPI, was dialyzed against PBS. In all experiments, the final concentration of Cof-22 was 1 μg/ml.

IgG fractions were isolated from the serum of one patient with primary APS and sera of two children with atopic dermatitis (AD), containing high titers of IgG anti-β_2GPI, by affinity purification on a 2 ml protein-G column (Pierce, Rockford, USA), using the protocol recommended by the manufacturer. As a control, IgG fraction from a normal human serum was purified by the same method. The IgG preparations were equilibrated against PBS, pH 7.4, in a desalting column. The IgG fractions from the patient with primary APS and two patients with AD gave comparable results in the anti-β_2GPI ELISA performed as described previously [26]. The levels of anti-β_2GPI in the IgG preparations were considered as medium range, amounting to about a half of the anti-β_2GPI levels in the original sera.

3. Results

3.1. Transfer of GPVs into the solution containing β_2GPI

Most GPVs transferred to the solution containing only β_2GPI at 100 μg/ml budded only moderately and about 30% of them did not form buds at all. Interestingly, more spherical GPVs formed less or no buds. In contrast, more laxed, flaccid GPVs, whose membranes were fluctuating more intensely, exhibited more pronounced budding. The average number of buds observed for 10 transfers in 5 different experiments was 1.5 to 2.5 per vesicle. No GPV branching into daughter vesicles was detected in any of the experiments. Most GPVs reached the steady state in 1 to 5 min. Further observation for up to 2 h did not reveal any additional changes. A representative experiment is shown in Fig. 1A–F.

3.2. Addition of Cof-22 into the solution containing β_2GPI and GPVs

After the addition of Cof-22 into the solution with β_2GPI and a single GPV, which already reached the steady state, additional budding started in 15 to 30 s (Fig. 2A–D).

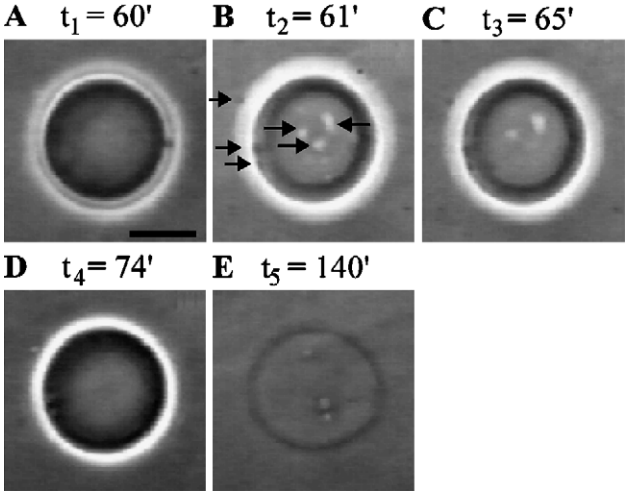

Fig. 2. Changes of GPVs following the addition of Cof-22. (A) GPV in a steady state after 60-min incubation in the solution containing β_2GPI (the bar indicates 20 μm). (B–D) Newly formed buds (arrows) following the addition of Cof-22 after a 60-min incubation. The number of buds diminished during observation due to the formation of daughter vesicles (not shown). (E) The GPV gradually faded and diminished in size during observation. (*t*: time in minutes (′) after transferring GPV into the test solution).

This secondary budding was more pronounced than that observed in the presence of β_2GPI only. The number of buds varied from 0 to more than 20, depending mostly on the flaccidity of the GPV's membrane. In about 60% of the experiments, where additional budding was observed, we could detect some buds further separating from GPV as daughter vesicles. The diameter of daughter vesicles was about 1–3 μm, and the diameter of the original GPVs decreased by up to 20%. Most GPVs reached the equilibrium in up to 5 min. However, after longer observation, some GPVs faded (5 to 50% of GPVs in different experiments). Fading was observed mostly in less flaccid, nearly spherical GPVs. The fading was a consequence of a decrease in the sucrose concentration inside GPVs indicating permeabilization of their membranes (Fig. 2E).

3.3. Transfer of GPVs into the solution containing β_2GPI and Cof-22

The budding was observed in more than 80% of GPVs transferred individually into the solution containing β_2GPI and Cof-22. The budding was mostly very intensive and many of GPVs generated also daughter vesicles. Up to one half of the GPVs faded due to a decrease in the sucrose concentration inside them. A representative experiment is presented in Fig. 3A–I. No such effects were observed when the solution contained only Cof-22 (Fig. 4A–C).

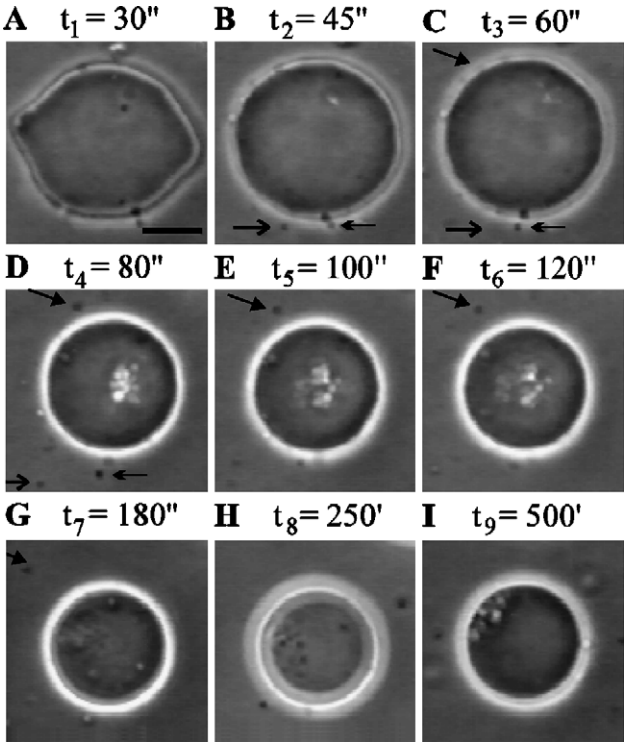

Fig. 3. Budding of GPVs transferred into the solution containing β_2GPI and Cof-22. (A) Intense budding of the GPV, which started quickly after the transfer (the bar indicates 20 μm). (B–G) Further budding of the GPV. Three different arrows indicate three small daughter vesicles separating and moving away from the GPV. (H, I) Steady state of the GPV reached after approximately 3 min (*t*: time in seconds (") after transferring GPV into test solution).

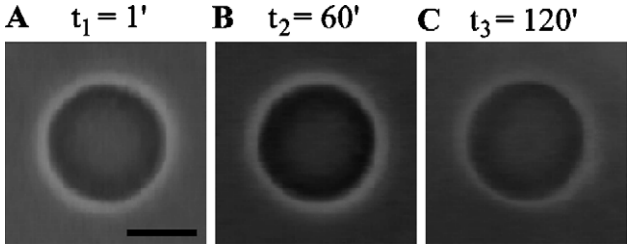

Fig. 4. Transfer of GPV into the solution containing Cof-22 without β_2GPI. (A–C) No changes were observed during a long observation of the GPV. The bar in the Panel A indicates 20 μm (*t*: time in minutes (′) after transferring GPV into the test solution).

3.4. Transfer of GPVs into the solution containing β_2GPI and the IgG fraction of a patient with primary APS

The effects were similar to those upon the addition of GPV to the solution containing β_2GPI and Cof-22. The budding of GPVs was mild to moderate and present in most GPVs, and some of them also generated daughter vesicles. Some of the GPVs also faded, but the effect was much less pronounced compared to the experiments with β_2GPI and Cof-22. No such effects were seen when the solution contained only the IgG fraction from a patient with primary APS without β_2GPI.

3.5. Transfer of GPVs into the solution containing β_2GPI and IgG fractions of children with atopic dermatitis (AD) or purified normal human IgG

The presence of the IgG fraction from either of the 2 children with AD or from the normal human serum in the solutions together with β_2GPI did not enhance the budding or fading of GPVs compared to the results obtained with β_2GPI alone.

4. Discussion

The interaction between polypeptides and membranes is a fundamental aspect of cell biochemistry. Liposomes have been frequently used in this context as in vitro system to study such interactions, however the influence of polypeptides on the shape of membranes cannot be reliably and easily studied by liposomes. We present here the case of giant phospholipid vesicles, which, due to their size (diameter larger than 10 μm), mimic more closely the situation observed in cell membranes and furthermore they permit to study the effect of protein–membrane interactions by direct optical monitoring. The in vitro experimental model employing giant phospholipid vesicles has already

been used for the study of some protein–phospholipid interactions [17,27,28]. The model with giant vesicles enables the observation of shape and changes of gray color intensity in a single GPV in real time. Our model with giant vesicles demonstrated also the dependence of the observed effect on the vesicle's state. Vesicles differed from one another at least in size and flaccidity, and after the transfer, their behavior was not fully predictable. The relative unpredictability of vesicles' behavior ruled out studies of the influence of small concentration changes of β_2GPI or anti-β_2GPI. However, we clearly observed the influence of β_2GPI and several effects of simultaneous addition of β_2GPI and anti-β_2GPI on giant vesicles, which were not observed in the absence of β_2GPI.

In studies of membrane traffic processes it was already demonstrated that direct interactions between proteins and lipid bilayers are important in the acquisition of high membrane curvature of membrane transport carriers such as tubules and vesicles [29]. A variety of proteins have been identified that directly bind and deform membranes [29]. Our results showing the budding of phospholipid vesicles in the presence of β_2GPI suggest that β_2GPI could also be involved in processes of budding and vesiculation of cellular membranes. Since β_2GPI is an abundant plasma protein, it binds to virtually every cell expressing negatively charged phospholipids in the outer layer of the cell membrane. Therefore, the involvement of β_2GPI in the budding and vesiculation of biological membranes is likely to be one of its physiologic functions, governing the curvature enhancement of lipid membranes as the basis of some cellular processes [30]. One possible example of β_2GPI involvement in cellular processes is the formation of apoptotic blebs on the cells loosing transmembrane lipid asymmetry at the beginning of apoptosis, where abundant quantities of β_2GPI were detected [31]. The budding of phospholipid vesicles could be explained by the insertion of the C-terminal loop of β_2GPI into the outer layer of a phospholipid membrane as previously described [6], which increases the area of the outer layer of the phospholipid bilayer. The change in the area of one membrane monolayer results, according to the bilayer couple hypothesis [32,33], in the transformation of the vesicles' shape. It was shown by experimental models that if the area of the outer monolayer became larger than the area of the inner one with a relative difference of about 1% or more, a small vesicle budded off a large vesicle [34,35]. Additionally, C-terminal loop of β_2GPI is relatively short and after insertion into a membrane it does not expand through the whole width of the outer layer of the membrane. Its insertion into an external portion of outer layer of the membrane thus necessarily causes also an increase of the spontaneous curvature of the membrane [36,37]. Summing up, the budding of vesicles after the insertion of C-terminal loop of β_2GPI into a membrane could be therefore explained by both the increased surface of outer layer and the increased spontaneous curvature of the membrane.

Although different pathogenic actions of anti-β_2GPI have been described by now [12,26], their role in promoting blood coagulation is not yet fully understood. The increased number of microparticles with procoagulant properties derived from the vesiculation of endothelial cells, platelets and other blood cells have been detected in patients with the APS, but the explanation for this finding has not yet been provided [13,14]. It was already shown that in the presence of anti-β_2GPI from patients with APS, the binding of β_2GPI to phospholipids is greatly enhanced due to the formation of stable complexes formed by two β_2GPI molecules adsorbed to a negatively charged membrane and one molecule of anti-β_2GPI bound to epitopes on β_2GPI [38]. In our experiments with both β_2GPI and anti-β_2GPI we observed more pronounced budding of GPVs than in experiments with β_2GPI alone. Additionally, when β_2GPI and anti-β_2GPI were simultaneously present in the solution, small daughter vesicles were formed on some GPVs. As shown in the experiments without β_2GPI, anti-β_2GPI alone cannot affect vesicle's shape in the absence of β_2GPI. Hence, we demonstrated that enhanced budding and vesiculation was the result of simultaneous action of β_2GPI and anti-β_2GPI and it could be explained by enhanced binding of β_2GPI to phospholipid membranes in the presence of anti-β_2GPI [38]. Since β_2GPI is an abundant plasma protein, the same situation can be present also in patients with anti-β_2GPI. We therefore concluded that more pronounced budding of membranes and formation of daughter vesicles is a feasible pathogenic action of anti-β_2GPI. It is reasonable to conclude that this pathogenic action of anti-β_2GPI might be the cause for increased number of microparticles with procoagulant properties derived from endothelial cells, platelets, and other blood cells as detected in patients with APS.

Previously, it was found that anti-β_2GPI may in vitro cause neuronal cell dysfunction [15,16] by an unknown mechanism. It is known, however, that the function of neuronal cells is very sensitive to small changes of membrane potential, which is heavily affected by the flow of solution across their membrane. Our results showed that anti-β_2GPI could cause in the presence of β_2GPI leaking of GPV membranes. The permeabilization of GPVs in the presence of β_2GPI and anti-β_2GPI Cof-22 observed in our experiments could be, similarly as the budding and vesiculation, explained by the insertion of the β_2GPI C-terminal loop into outer layer of phospholipid membranes. The permeabilization and consequent fading of GPVs was present predominantly in more spherical GPVs. The volume of these GPVs was very close to the maximum volume that could be reached with a finite membrane area. Theoretically, as the mean shape of a vesicle approaches a sphere, the thermal shape fluctuations become smaller, since for a sphere as a stationary shape, there is no space available for fluctuations at the constant volume [39]. In the case of a nearly spherical vesicle, when additional molecules of β_2GPI were inserted into the outer monolayer the increased difference between the monolayer areas

instead of causing a shape change rather causes an increased tension in membrane monolayers, which can trigger the formation of a transient pore and consequent leaking of the membrane [17].

The above described mechanism might give the explanation for the effects of anti-β_2GPI on neuronal cells. The affection of central nervous system is commonly observed in patients with APS [40]. Antiphospholipid antibodies including anti-β_2GPI were most frequently associated with cerebrovascular disease. Other neurological syndromes, including dementia, migraine, chorea, transverse myelopathy, Guillain Barre syndrome, transient global amnesia, seizures, motor neuron disease, myasthenia gravis, and depression, have also been described in patients with APS [40]. Although some of them (for example, dementia, chorea, seizures, and transient global amnesia) could well be the result of the cerebrovascular disease, the relationship of anti-β_2GPI to the underlying pathophysiology of other syndromes is less clear pointing to the non-thrombotic effect of anti-β_2GPI [41]. Since we showed that anti-β_2GPI in the presence of β_2GPI can cause permeabilization of a phospholipid membrane, our result supports the hypothesis about a direct action of anti-β_2GPI on some cell types. This could be a novel non-thrombotic pathogenic action of anti-β_2GPI, observed especially in nervous cells.

Opposite to Cof-22 that binds to domain III, and anti-β_2GPI from patients with APS recognizing most probably domains I and IV of β_2GPI [42,43], anti-β_2GPI from children with AD did not enhance the budding and did not cause the permeabilization of GPVs. The most likely reason for the lack of such an effect was the different epitopic specificity of anti-β_2GPI in AD, which recognize an epitope at the phospholipid binding site or very close to the phospholipid binding site on domain V of β_2GPI [26]. Due to their epitope specificity, anti-β_2GPI from children with AD could not bind β_2GPI associated with phospholipid membrane because of sterical hindrance of the phospholipid membrane [26]. We can explain the lack of vesicles' budding in the presence of anti-β_2GPI directed to domain V by the inability of these type of anti-β_2GPI to enhance the binding of β_2GPI to phospholipids as described for anti-β_2GPI from patients with APS recognizing other domains of β_2GPI [38].

In conclusion, we described for the first time the interactions of β_2GPI, anti-β_2GPI and GPVs in a simple in vitro model, excluding most external influences. We pointed to a possible physiologic role of β_2GPI in the budding of phospholipid membranes expressing negatively charged phospholipids on the outer surface. Furthermore, we demonstrated an enhanced budding and vesiculation of phospholipid membranes by anti-β_2GPI, which could explain the generation of increased quantities of prothrombotic microparticles in patients with APS as a feasible prothrombotic action of anti-β_2GPI. Additionally, a possible non-thrombotic mechanism of anti-β_2GPI action was described for the first time.

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