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THROMBOMODULATORY EFFECT OF ANTI-B_2-GLYCOPROTEIN I ANTIBODIES ON CRYSTALLINE ANNEXIN A5 ON PHOSPHOLIPID BILAYERS, AS OBSERVED BY ATOMIC FORCE MICROSCOPY

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Abstract

Antibodies against β2-glycoprotein I are a subset of very heterogeneous family of antiphospholipid antibodies. It is well recognised that anti-β2-glycoprotein I antibodies are the main pathogenic players in the autoimmune disease known as antiphospholipid syndrome. Many mechanisms have been proposed through which these autoantibodies could cause microplacental, arterial or venous thrombosis. One of the suggested mechanisms is an antiphospholipid antibody-mediated disruption of annexin A5 protective crystalline shield on negatively charged phospholipid membranes. In current report the study of β2-glycoprotein I, anti-β2-glycoprotein I antibodies and annexin A5 interactions was performed on in vitro model of planar solid-supported phospholipid bilayers and visualized by atomic force microscopy. Planar phospholipid bilayers comprised 30 % L-α-phosphatidylserine and 70 % L-α-phosphatidylcholine. For the study of interactions 10 mg/l annexin A5, 0.15 g/l β2-glycoprotein I, 10 g/l of IgG fraction from healthy blood donor, 10 g/l of IgG fraction from a patient with anti-β2-glycoprotein I antibodies and 0.4 g/l of isolated IgG anti-β2-glycoprotein I antibodies from the same patients in Hepes buffered saline with 1.5 mM Ca2+ were used. We confirmed the clustering of β2-glycoprotein I on planar phospholipid bilayers. We also found that in the presence of annexin A5, β2-glycoprotein I does not bind to planar phospholipid bilayers. However, when adding the anti-β2-glycoprotein I antibodies, the growth of β2-glycoprotein I-anti-β2-glycoprotein I antibodies complexes in the presence of incompletely crystallized annexin A5 on planar phospholipid bilayers was observed. Results confirm the possible thrombomodulatory activity of anti-β2-glycoprotein antibodies through their effect on crystalline annexin A5. In addition, the hypothesis that the presence of possibly pathologic antigen-antibody pair itself is not sufficient to start the pathological process is confirmed and visualized for the first time.

Abbreviations: AFM, atomic force microscopy; ANX A5, annexin A5; aPL, antiphospholipid antibodies; APS, antiphospholipid syndrome; anti-β2-GPI, anti-β2-glycoprotein I antibodies; HBS-Ca2+, Hepes buffered saline with

calcium; L-α-PC, L-α-phosphatidylcholine; L-α-PS, L-α-phosphatidylserine; SPB, solid supported phospholipid bilayer; β2-GPI, β2-glycoprotein I.

INTRODUCTION

Antiphospholipid antibodies (aPL) represent laboratory criterium in addition to clinical criteria for determining the antiphospholipid syndrome (APS) [1 - 4]. APS is an enigmatic autoimmune condition for which several different thrombogenic and non-thrombogenic pathological mechanisms have been proposed, but not conclusively established [5 - 7]. One of such suggested mechanisms is aPL-mediated disruption of the annexin A5 (ANX A5) anticoagulant shield that could be the reason for development of micro placental, venous or arterial thrombosis in patients with APS [8, 9].

Among the most studied aPL are anti-β2-glycoprotein I antibodies (anti-β2-GPI). Anti-β2-GPI are directed against protein cofactor β2-glycoprotein I (β2-GPI). β2-GPI is a glycoprotein of 54 kDa, with plasma concentration of about 150 mg/l. The crystal structure of the protein reveals five domains joined like beads on a string to form a circular conformation or an open elongated J-shaped molecule conformation [10, 11, 12]. Each domain consists of 60 amino acids, except for domain V. Domain V consists of 82 amino acids due to C-terminal extension of 19 amino acids and an insertion of 6 amino acids, forming a hydrophobic loop. In addition, domain V carries a definite positive charge arising from 14 lysine residues. These specific structures of domain V are responsible for interconnection of domains V and I of the molecule to form a circular confirmation [12]. In addition, β2-GPI binds to anionic phospholipids in an open J-shaped conformation through the domain V [13 - 16]. Upon binding to negatively charged phospholipid surfaces, conformation of β2-GPI changes and oligomerization (clustering) of the protein molecules on the phospholipid surface occurs [17].

Many theories have been proposed to explain the interaction of anti-β2-GPI to β2-GPI [12, 18-21]. Anti-β2-GPI of different isotypes, directed toward different epitopes of the β2-GPI molecule and of different avidity, have been associated with various clinical manifestations [22]. It has become clear that IgG isotype, directed toward the domain I of β2-GPI and of high avidity, is connected with thrombosis and pregnancy complications in patients with APS [9, 23, 24-27]. On the other hand, anti-β2-GPI of lower avidity have been described in patients with systemic lupus erythematosus [25]. Domain IV and domain V have also been described as target sites for some anti-β2-GPI; however, thrombosis has not been associated with the occurrence of these subtypes of autoantibodies [28-30]. IgM isotype against the domain V of the antigen was detected in infectious diseases [31] and childhood atopic dermatitis [32]. IgA isotype that recognises domains IV and V of the β2-GPI has been determined in patients with stroke and acute coronary syndrome [33].

The mechanisms by which anti-β2-GPI contribute to pathogenesis of the before mentioned conditions are elusive. Binding of the complex of β2-GPI and anti-β2-GPI to throphoblast cells [34] was shown to cause activation of the cells and down-regulation of trophoblast human chorionic gonadotropin synthesis and its secretion. The complex was also found to promote the tissue factor-induced thrombin generation in the presence of tissue factor pathway inhibitor [35]. There is evidence that complex of β2-GPI- anti-β2-GPI may activate endothelial cells and monocytes through binding to Toll-like receptor-4, thereby stimulating the innate (unspecific) immunity [36]. Toll-like receptors are receptors that induce prompt inflammatory responses and mediate functional activation in immune effector cells. However, among the suggested thrombogenic pathological mechanisms, the aPL-mediated disruption of ANX A5 protective crystalline shield over the phospholipid surfaces should be emphasised [8, 9]. ANX A5 is a 36 kDa water-soluble protein that in the presence of calcium binds to negatively charged phospholipid surfaces [37, 38]. The protein is formed of four domains consisting solely of α-helices, and is shaped like concave disk with calcium and phospholipid-binding domains present on the convex side [39]. ANX A5 molecules are monomeric in solution, however, after binding to phospholipid membrane, they spontaneously form tightly bound trimers. The membrane bound trimers self-organise into two types of 2D crystals, with p3 and/or p6 symmetry [40]. The anticoagulant

properties of the protein are a consequence of ANX A5 crystallization on phospholipid membranes, resulting in a lattice of protein over phospholipid surfaces. This blocks the availability of phospholipid surfaces for coagulation reactions. It has previously been demonstrated that larger quantities of ANX A5 are released from the apical surfaces of placental syncytiotrophoblast and umbilical vein endothelial cells [41]. This places the protein in the anatomic position where pathological consequences of aPL take place and therefore, confirms its proposed anticoagulant protective role. In addition, the anticoagulant activity has been connected to more specific properties of ANX A5, such as down-regulation of surface-expressed tissue factor [42]. The phenomenon of aPL-mediated disruption of ANX A5 protective shield, has been studied by different experimental groups, using various techniques (immunoassay, ellipsometry, atomic force microscopy (AFM), fluorescence light microscopy and flow cytometry system) and all three basic (in vitro, ex vivo and in vivo) experimental models [9, 24, 43-47].

Despite being the most extensively studied subset of aPL, anti-β2-GPI characteristics are somewhat ambiguous. The present study was performed to give visual insight into one of the proposed pathogenic mechanisms i.e. the effects of anti-β2-GPI on ANX A5 protective crystalline shield on negatively charged phospholipid surfaces in the presence of β2-GPI. Naturally derived reagents (phospholipids, ANX A5, β2-GPI and antibodies) were employed to ensure relevance of the study to the physiological conditions in human body. The interactions of ANX A5, β2-GPI and anti-β2-GPI were studied on in vitro model of planar solid-supported phospholipid bilayers, using AFM. AFM, allows “in situ” visualization of the addressed interactions in a nanomolecular scale. The knowledge about molecular interactions is prerequisite for the improvement of laboratory diagnostics and more targeted therapeutic approaches of patients with anti-β2-GPI.

MATERIALS AND METHODS

Planar solid-supported phospholipid bilayers (SPBs) were prepared on mica, simulating the physiological conditions of the cell surface. On SPBs, binding of β2-GPI and isolated antibodies (IgG fraction of patients with anti-β2-GPI, isolated anti-β2-GPI from the same patient and IgG fraction from a healthy blood donor) were studied in the absence or presence of ANX A5. The effects were visualized by imaging using AFM.

1. HUMAN SERA, AFFINITY PURIFICATION OF IGG AND ISOLATION OF ANTI-B2 -GPI

Sera were selected from the sera bank of the Department of Rheumatology (University Medical Centre, Ljubljana, Slovenia). Sera were pre-tested by an in-house or commercial enzyme-linked immunosorbent assay for the presence of different subtypes of aPL. The aPL tested were: anti-cardiolipin antibodies [48], anti-β2-GPI antibodies [49], anti-prothrombin antibodies [50, 51] and anti-annexin A5 antibodies (Orgentec Diagnostica, Mainz, Germany). In the present study, sera were selected according to clinical features of the patient, immunologic tests and availability (adequate volume stored). Total IgG from selected sera were purified with MAbTrapTM Kit (Amersham, GE Healthcare, Little Chalfont, UK), according to manufacturer’s instructions. After purification, the preparations were dialysed against Hepes buffered saline (HBS) (10 mM HEPES, 150 mM NaCl), containing 1.5 mM calcium (HBS-Ca2+), pH 7.5. HBS-Ca2+ was prepared in water for injections (Braun, Melsungen, Germany). Part of isolated IgG, after affinity purification with MAbTrapTM Kit, were dialysed against Phosphate buffered saline (PBS) and then further subjected to isolation of anti-β2-GPI by an in-house affinity column [in detail in 40]. Isolated anti-β2-GPI were dialysed against HBS-Ca2+. Concentrations of purified proteins were determined spectrophotometrically (Camspec M501 Single Beam Scanning UV/Visible Spectrophotometer, Camspec Ltd., Cambridge, UK), using the extinction coefficient of 14.0 for the 1% IgG solution. Obtained samples were designated: i.) sample IgGA, 10 g/l, IgG fraction from a patient A with primary APS who suffered from venous thrombosis and was positive for IgG anti-cardiolipin antibodies and anti-β2-GPI; ii.) sample anti-β2-GPIA, 0.4 g/l, isolated anti-β2-GPI from a patient A, IgG isotype; and iii.) sample IgGB, 10 g/l, IgG fraction from a healthy blood donor without aPL.

2. PREPARATION OF SPB ON MICA

L-α-phosphatidylserine (L-α-PS) (Sigma- Aldrich, St. Luis, MO, USA), 30% w/w, and L-α-phosphatidylcholine (L-α-PC) (Sigma- Aldrich, St. Luis, MO, USA), 70 % w/w, were dissolved in chloroform. Solvent was evaporated under low

pressure in the presence of N2 for at least 30 min. HBS-Ca2+ was added to the obtained lipid film, yielding a 0.5 mg/ml multilamellar vesicle suspension. Unilamellar vesicles were obtained using a bath sonicator (UZ 4R, Iskra, Kranj, Slovenia). SPB was formed by deposition of 120 µl of freshly sonicated vesicles onto a cleaved mica surface followed by incubation for 60 min at room temperature. The sample was further heated to 41°C for 10 min. The excess of lipids was removed by exchanging the solution covering mica with buffer, and the sample was then installed in the contact mode fluid cell in the AFM. The microscope was allowed to thermally equilibrate for a minimum of 15 min before imaging. The presence of SPB was confirmed by imaging and force measurements [in detail in 52].

3. Binding of β2 -GPI and isolated antibodies to SPBs

After the presence of SPB was confirmed, β2-GPI (0.15 g/l in HBS-Ca2+), purified from pooled human plasma [in detail in 55]) or/and isolated antibodies (IgGA, IgGB, anti-β2-GPIA) were injected into the fluid cell of the AFM. Effects of β2-GPI or/and antibodies on SPBs were measured by AFM for 60 minutes.

4. Binding of β2 -GPI to SPBs in the presence of ANX A5

After the presence of SPB was confirmed, 10 mg/l ANX A5 isolated from human placenta (Sigma- Aldrich, St. Luis, MO, USA) in HBS-Ca2+ was injected into the fluid cell of the AFM. When the presence of ANX A5 crystalline shield, completely or incompletely covering the SPB was detected, β2-GPI (0.15 g/l in HBS-Ca2+) was added to the ANX A5 solution covering the SPB. ANX A5 concentration was kept at 10 mg/l. Results from the experiment where applying β2-GPI before the ANX A5 to SPBs, were also resolved. The binding of β2-GPI and ANX A5 on SPBs were measured by AFM for 60 minutes.

5. Effects of isolated antibodies on crystalline ANX A5 on SPBs

When the presence of ANX A5 crystalline shield, completely or incompletely covering the SPB was detected, antibodies (IgGA, IgGB, anti-β2-GPIA) were added to the ANX A5 solution covering the SPB. ANX A5 concentration was kept at 10 mg/l. Results from the experiment where applying antibodies before the ANX A5 to SPBs, were also resolved. The effects of antibodies on crystalline ANX A5 were measured by AFM for 60 minutes.

6. Effects of β2 -GPI and isolated antibodies on crystalline ANX A5 on SPBs

The main focus of our survey were the effects of β2-GPI-anti-β2-GPI pair on completely and incompletely crystallized ANX A5 on SPBs. When the presence of ANX A5 crystalline shield, completely or incompletely covering the SPB was detected, β2-GPI (0.15 g/l in HBS-Ca2+) and antibodies (IgG A, IgG B, anti-β2-GPIA) were added to the ANX A5 solution covering the SPB. ANX A5 concentration was kept at 10 mg/l. Results from the experiment where applying antigen-antibody pair before the ANX A5 to SPBs, were also resolved. The effects of β2-GPI and antibodies together on crystalline ANX A5 were measured by AFM for 60 minutes.

7. Atomic Force Microscopy

AFM measurements were performed in a liquid environment using a Nanoscope IIIa-MultiMode AFM (Digital Instruments, Santa Barbara, CA) equipped with the E (15µm) scanner. Mica substrates were installed into a contact mode fluid cell. Using AFM we have measured forces and acquired images in the constant force mode using oxide-sharpened silicon nitride tips (MSCT-AUNM silicon nitride tip, Veeco, Camarillo, CA) mounted on cantilevers with nominal force constant of 0.01 N/m or 0.03 N/m, with typical curvature radius of 15 nm. We used different scanning rates, most often 5 Hz. The scanning force was kept at the lowest possible value by continuously adjusting the set point during imaging. All experiments were performed at 25°C.

Results

1. Binding of B_2 -GPI and isolated antibodies to SPBs

When studying binding of β_2-GPI to continuous SPBs, we found that the protein binds to phospholipid bilayers by forming protein agglomerates (clusters) over them (see **Figure 1 A, B, C**). Protein clusters were measured 3.1 ± 0.2 nm (average ± SD) higher than phospholipid surface on which they were formed. SPBs were measured 3.6 ± 0.4 nm higher than mica [in detail in 58]. If antibody sample containing anti-β_2-GPI (IgG A or anti-β_2-GPIA) was added, anti-β_2-GPI bound to the antigen clusters (Figure 1 D, E). The observed antigen-antibody patches expanded to almost all the observed phospholipid surface and rose in height to approximately 13 nm above the SPB. However, no binding on β_2-GPI clusters on SPB was measured for control sample IgG B, as expected. None of the studied antibodies (IgG A, IgG B, anti-β_2-GPIA) bound to SPBs on their own.

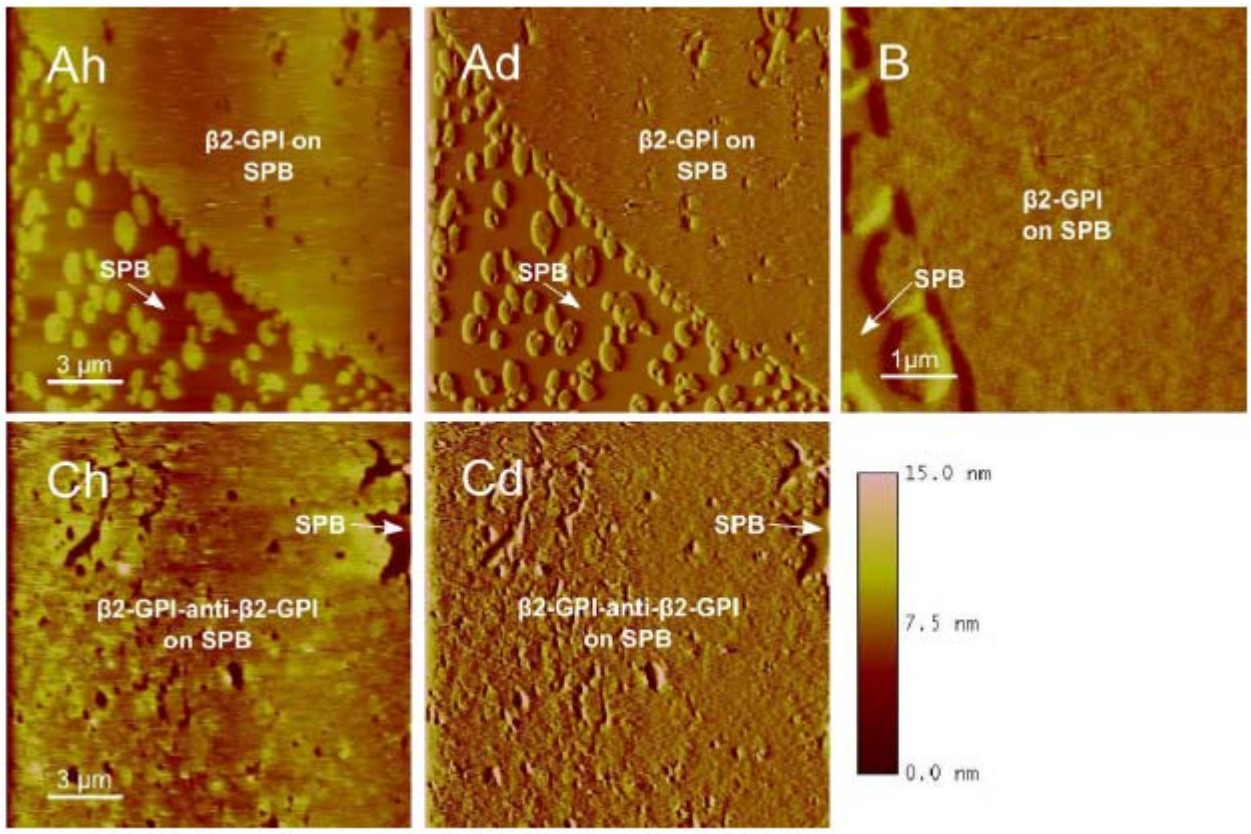

FIGURE 1: AFM HEIGHT AND DEFLECTION IMAGES OF BINDING B2-GPI AND SAMPLE IGGA TO CONTINUOUS SPB. AH.) HEIGHT IMAGE SHOWING B2-GPI (0.15 G/L IN HBS-CA2+) CLUSTERS ON SPB, MEASURED APPROXIMATELY 3.1 NM HIGHER THAN PHOSPHOLIPID SURFACE ON WHICH THEY ARE FORMED. AD.) DEFLECTION IMAGE OF THE SAME SURFACE AS IN AH. B.) ZOOMED DEFLECTION IMAGE OF THE PROTEIN CLUSTER ON SPB. SOME ORDERED STRUCTURE CAN BE OBSERVED. AFTER ADDITION OF IGGA (10 G/L IN HBS-CA2+), ANTIBODIES (NAMELY ANTI-B2-GPI) BIND TO THE B2-GPI CLUSTERS ON SPB. CH.) HEIGHT IMAGE OF ANTIGEN-ANTIBODY PATCHES, THAT EXPANDED TO ALMOST ALL THE OBSERVED SURFACE AND ROSE IN HEIGHT TO APPROXIMATELY 13 NM ABOVE SPB. CD.) DEFLECTION IMAGE OF THE SAME SURFACE AS IN CH.

We found, that if mica surface is exposed (e.g. in the case of discontinuous SPBs, where SPBs is not covering the whole mica surface) β2-GPI preferably binds to mica than SPBs. The patches of β2-GPI on mica were measured 5 ± 0.5 nm higher than SPBs. If sample that contains anti-β2-GPI (IgG A or anti-β2-GPIA) was added to β2-GPI patches on mica, patches rose in height to around 15 nm above the SPBs, but did not expand. No binding to β2-GPI patches on mica was measured for a control sample IgG B.

In another set of experiments antibodies (IgG A, anti-β2-GPIA or IgG B) were administered to discontinuous SPB first (Figure 2). Antibodies bound to mica. Antibody patches 5 ± 2 nm higher than SPB were measured. No binding of

antibodies to SPBs was detected. However, if then β2-GPI was added, the antibody patches on mica raised in heights to about 12 nm above SPBs. β2-GPI bound to SPBs and if anti-β2-GPI were present in the studied sample, the anti-β2-GPI bound to β2-GPI on SPBs. Those antigen-antibody patches on SPBs expanded to cover the SPB. They were measured approximately 13 nm higher than SPBs.

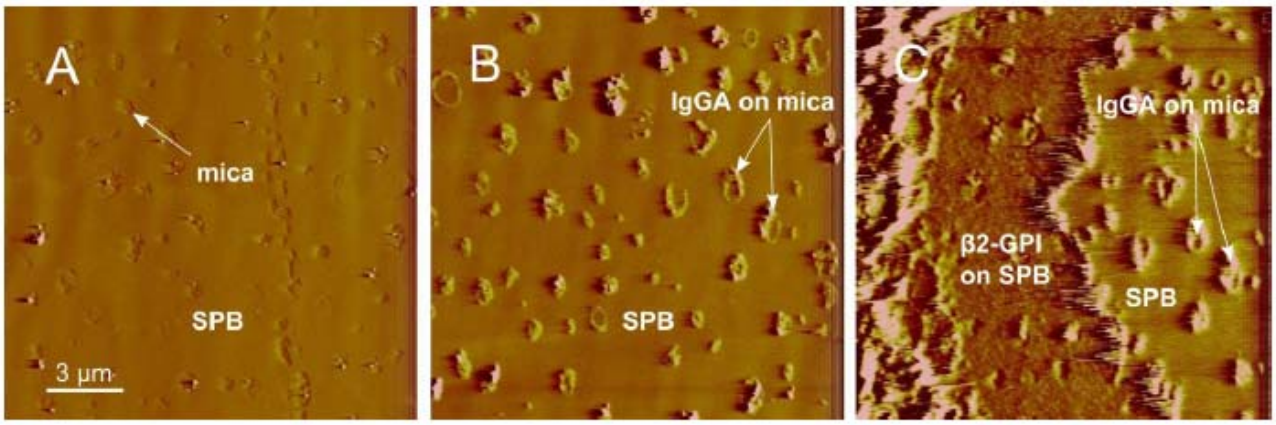

FIGURE 2: AFM DEFLECTION IMAGES OF BINDING IGGA AND B2-GPI TO DISCONTINUOUS SPB. A.) IMAGE OF DISCONTINUOUS SPB (SPB NOT COVERING THE WHOLE MICA SURFACE). B.) AFTER ADDITION OF IGGA (10 G/L IN HBS-CA2+), ANTIBODIES BIND TO MICA WHERE EXPOSED. NO BINDING OF ANTIBODIES TO SPB CAN BE DETECTED. C.) AFTER ADDING B2-GPI (0.15 G/L IN HBS-CA2+) IN THE SYSTEM, THE GLYCOPROTEIN BINDS TO SPB. DEFLECTION IMAGE IS SHOWING B2-GPI CLUSTERING ON SPB.

2. Binding of B_2-GPI to SPBs in the presence of ANX A5 and its effect on crystalline ANX A5 on SPBs

Since β_2-GPI and ANX A5 both bind to negatively charged phospholipid membranes, the binding of each in the presence of the other was studied. When studying binding of ANX A5 to SPBs, ANX A5 crystalline domains were 2.6 ± 0.2 nm higher than the lipid surface [52]. In our study model with SPBs containing 30% of phosphatidylserine and 10 mg/l of ANX A5 in HBS with 1.5 mM calcium, p6 crystal form was predominantly found. We found that β_2-GPI did not bind to SPBs in presence of ANX A5, even if ANX A5 did not crystallize over the whole SPBs surface (incompletely crystallized ANX A5). The β_2-GPI bound to mica where exposed, forming patches 2 ± 0.5 nm higher than crystalline ANX A5 on SPBs. If the β_2-GPI was administered to the SPBs before ANX A5, β_2-GPI bound to SPBs, but after addition of ANX A5 it desorbed from SPBs and the growth of ANX A5 crystalline domains was deduced.

3. Effects of antibodies on crystalline ANX A5 on SPBs

No effect of isolated antibodies (IgG A, IgG B or anti-β_2-GPIA) on completely or incompletely crystallized ANX A5 on SPBs was detected. Antibodies bound only to mica, where exposed. Antibody patches on mica were measured 3 ± 2 nm higher than crystalline ANX A5 on SPBs.

4. Effects of B_2-GPI-IgGA pair on crystalline ANX A5 on SPBs

Study of the interactions between β_2-GPI, anti-β_2-GPI and ANX A5 on phospholipid bilayers is the main focus of our research. We found no effect nor binding of β_2-GPI-IgGA pair to SPBs when completely and incompletely crystalline ANX A5 was present (IgG A contains anti-β_2-GPI). The sequence of addition of reagents (ANX A5, β_2-GPI and IgG A) into the AFM fluid cell, to SPBs, had no effect on the result. In **Figure 3** the results from adding first ANX A5, second β_2-GPI and third IgG A to discontinuous SPB are represented. ANX A5 crystallized on SPB and β_2-GPI and/or antibodies bound to mica, where exposed.

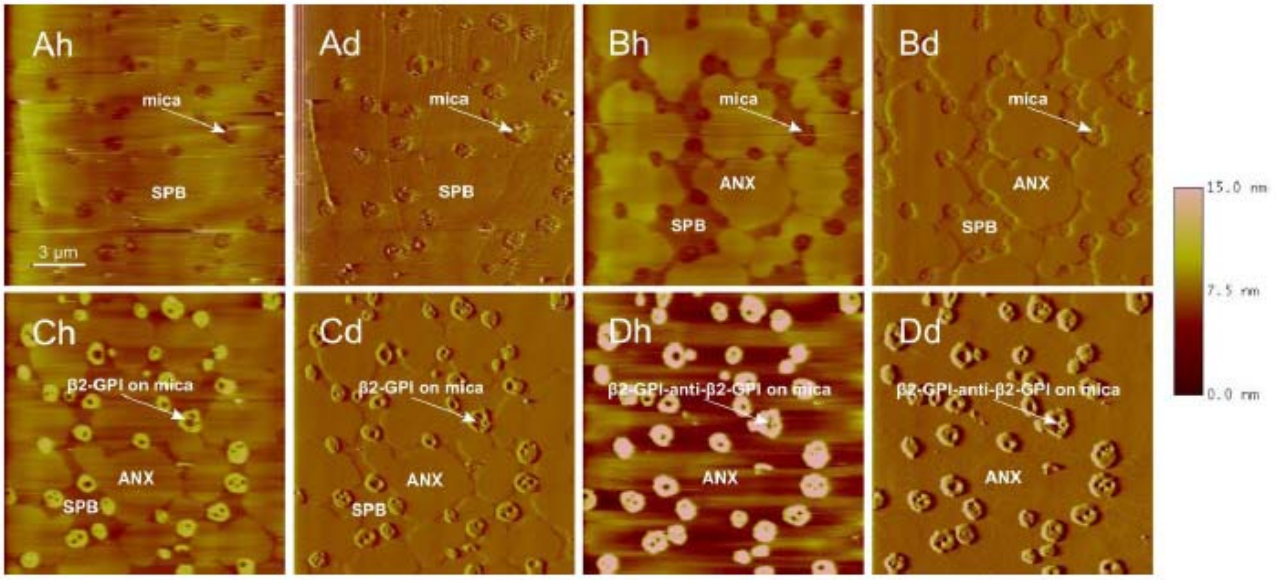

FIGURE 3: AFM HEIGHT AND DEFLECTION IMAGES OF THE EFFECT OF B2-GPI-IGGA PAIR ON INCOMPLETELY CRYSTALLIZED ANX A5 ON SPBS. AH.) HEIGHT IMAGE OF DISCONTINUOUS SPB, MEASURED APPROXIMATELY 3.6 NM HIGHER THAN MICA. AD.) DEFLECTION IMAGE OF THE SAME SURFACE AS IN AH. BH.) AFTER ADDITION OF ANX A5 (10 MG/L IN HBS-CA2+), GROWTH OF ANX A5 CRYSTALLINE DOMAINS ON SPB CAN BE OBSERVED. THE DOMAINS ARE APPROXIMATELY 2.1 NM HIGHER THAN SPBS. BD.) DEFLECTION IMAGE OF THE SAME SURFACE AS IN BH. CH.) BEFORE CRYSTALLINE ANX A5 COMPLETELY COVERED THE SPBS, B2-GPI (0.15 G/L IN HBS-CA2+) IS ADDED TO THE ANX A5 SOLUTION COVERING THE SPBS. B2-GPI BINDS TO EXPOSED MICA, FORMING PATCHES APPROXIMATELY 2 NM HIGHER THAN CRYSTALLINE ANX A5 ON SPBS. NO BINDING OF B2-GPI TO SPBS IS OBSERVED, WHEN ANX A5 PRESENT. CD.) DEFLECTION IMAGE OF THE SAME SURFACE AS IN CH. DH.) IGGA (10 G/L IN HBS-CA2+) IS ADDED TO THE ANX A5 AND B2-GPI SOLUTION COVERING THE SURFACE. ANTIBODIES BIND TO B2-GPI PATCHES ON MICA. PATCHES OF THE B2-GPI-IGGA COMPLEX ON MICA ARE APPROXIMATELY 13 NM HIGHER THAN CRYSTALLINE ANX A5 ON SPB. NO BINDING OF THE B2-GPI-IGGA COMPLEX TO SPBS IN PRESENCE OF ANX A5 IS DETECTED. DD.) DEFLECTION IMAGE OF THE SAME SURFACE AS IN DH.

5. Effects of B_2-GPI-anti-B_2-GPIA pair on crystalline ANX A5 on SPBs

The growth of β_2-GPI-anti-β_2-GPI patches on SPBs was detected when adding the β_2-GPI and anti-β_2-GPIA to incompletely crystallized ANX A5 on SPBs (anti-β_2-GPIA contains isolated anti-β_2-GPI of IgG isotype), as shown in **Figure 4.** Antigen-antibody patches on SPBs were measured 14 - 16 nm and crystalline ANX A5 2 - 3 nm higher than SPBs. The β_2-GPI-anti-β_2-GPI complexes bound to exposed mica, in addition to binding to SPBs. The patches on mica were measured 13 - 15 nm higher than SPB, but did not expand. However, when adding the β_2-GPI and anti-β_2-GPIA to completely crystallized ANX A5 on SPB, binding of antigen-antibody patches on SPB was not detected.

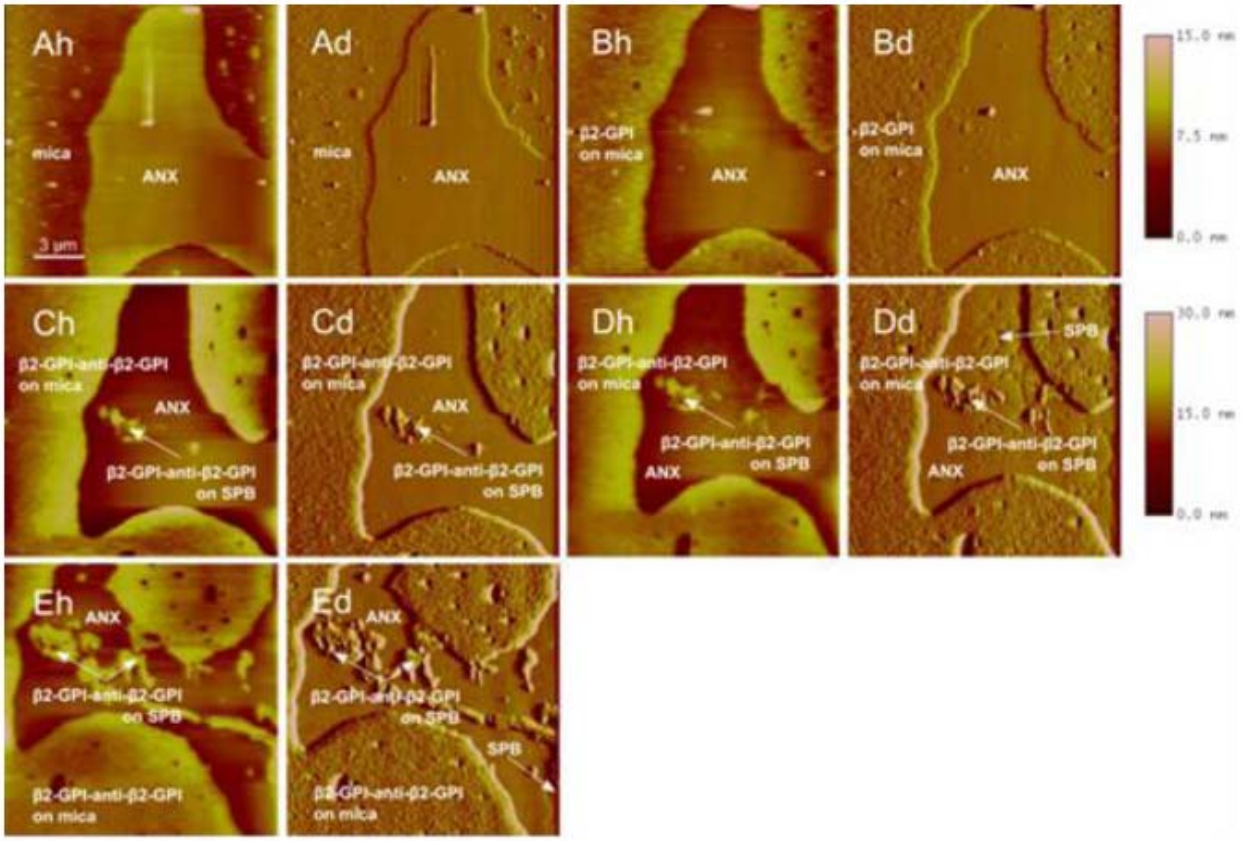

FIGURE 4: AFM HEIGHT AND DEFLECTION IMAGES OF THE EFFECT OF B2-GPI AND HIGH AVIDITY ANTI-B2-GPI ON CRYSTALLINE ANX A5 ON SPBS. AH.) HEIGHT IMAGE OF CRYSTALLINE ANX A5 (10 MG/L IN HBS-CA2+) ON SPB. THE SPB WITH CRYSTALLIZED ANX A5 IS MEASURED 5 - 6 NM HIGHER THAN MICA. AD.) DEFLECTION IMAGE OF THE SAME SURFACE AS IN AH. AFTER ADDITION OF B2-GPI (0.15 G/L IN HBS-CA2+) TO ANX A5 SOLUTION COVERING THE SURFACE, THE GLYCOPROTEIN BINDS TO MICA. BH.) HEIGHT IMAGE OF B2-GPI CLUSTERS ON MICA, 2 - 4 NM HIGHER THAN CRYSTALLINE ANX A5 ON SPB. BD.) DEFLECTION IMAGE OF THE SAME SURFACE AS IN BH. AS THIRD, THE ANTI-B2-GPIA WERE ADDED. CH.) HEIGHT IMAGE OF ANTIBODIES BOUND TO B2-GPI, FORMING PATCHES WITH HEIGHTS 12 - 14 NM ABOVE CRYSTALLINE ANX A5. IN ADDITION TO SIMPLE BINDING TO ALREADY BOUND ANTIGEN ON MICA, THE ANTIBODIES AND THE ANTIGEN FORM A COMPLEX WITH APPARENTLY SUFFICIENT AFFINITY TO BIND TO SPB EVEN IN THE PRESENCE OF ANX A5. THESE NEW PATCHES OF ANTIGEN-ANTIBODY COMPLEX ON SPB ARE 14 - 16 NM HIGHER THAN CRYSTALLINE ANX A5. CD.) DEFLECTION IMAGE OF THE SAME SURFACE AS IN CH. AFTER DESORPTION OF THE CRYSTALLINE ANX A5 OF SPB BY HBS-CA2+ BUFFER ALONE, THE MIXTURE OF ALL THREE REAGENTS WAS ADDED AGAIN. DH.) HEIGHT IMAGE OF THE LATERAL GROWTH OF B2-GPI- HIGH AVIDITY ANTI-B2-GPI PATCHES IS MEASURED. DD.) DEFLECTION IMAGE OF THE SAME SURFACE AS IN DH. THE PROCESS OF DESORPTION AND ADDITION OF THE MIXTURE WAS REPEATED. EH.) HEIGHT IMAGE OF THE LATERAL GROWTH OF B2-GPI- HIGH AVIDITY ANTI-B2-GPI PATCHES IS MEASURED AGAIN. THE VERTICAL HEIGHT OF THESE COMPLEXES IS STILL 14-16 NM ABOVE THE CRYSTALLINE ANX A5. ED.) DEFLECTION IMAGE OF THE SAME SURFACE AS IN EH.

6. EFFECTS OF B_2 -GPI-IGG B CONTROL PAIR ON CRYSTALLINE ANX A5 ON SPBS

The IgG B, which were isolated from a healthy blood donor, do not contain anti-β_2-GPI. Indeed no binding of IgG B to β_2-GPI was detected in our study. In addition, no effect on crystalline ANX A5 was found. The sequence of addition of reagents into the AFM fluid cell to SPBs had no effect on the result. ANX A5 crystallized on SPBs, β_2-GPI and IgG B (not in the form of complexes) bound to mica, where exposed.

DISCUSSION

Anti-β_2-GPI have been implicated with clinical symptoms of APS, namely thrombosis and pregnancy complications [2-4]. However, the mechanisms by which these autoantibodies participate in the pathology of APS have not yet been clearly established. In the nineties Rand et al introduced the hypothesis of aPL-mediated disruption of ANX A5 protective shield on phospholipid surfaces [8,9]. Our study offers visualization of the proposed pathological mechanisms of β_2-GPI, anti-β_2-GPI and ANX A5 on phospholipid surfaces, “in situ” and in a nanomolecular scale. In order to ensure physiological relevance of our *in vitro* model, naturally derived reagents (phospholipids, β_2-GPI,

antibodies and ANX A5) in their physiological concentrations were used. We confirmed the clustering of β_2-GPI on SPBs that could be enlarged by addition of anti-β_2-GPI. We found that in the presence of ANX A5, β_2-GPI does not bind to SPBs. Addition of ANX A5 in fact caused desorption of the pre bound β_2-GPI. When studying the antibodies (IgG A, IgG B, anti-β_2-GPIA), no binding to SPBs could be observed. Antibodies preferably bound to mica, when exposed. In addition, no effect on crystalline ANX A5 by antibodies on their own was found. However, when putting all three reagents ANX A5, β_2-GPI and antibodies together, the growth of β_2-GPI-anti-β_2-GPI complexes on SPBs in the presence of incompletely crystallized ANX A5 on SPB was observed. These results confirm the proposed thrombomodulatory effect of β_2-GPI-anti-β_2-GPI pair through its effect on crystalline ANX A5.

In our *in vitro* study, SPBs from naturally derived phospholipids with 30 % of L-α-PS in the mixture of L-α-PS and L-α-PC were used to mimic the cell membrane surface [52]. When studying binding of β_2-GPI to SPBs, supramolecular assemblies of the protein on natural SPBs with heights of about 3.1 nm were measured. Our results are in agreement with a previous study made on SPBs of synthetic phospholipids [17]. However, the measured heights are lower than expected from dimensions of the β_2-GPI molecule (13.2 x 7.2 x 2.0 nm^3 [11]) and suggest horizontal-like orientation of the molecule on SPBs. The horizontal orientation was confirmed in an extensive study by Hamdan et al [13], who showed the participation of not only domain V but also domains IV and III in insertion of β_2-GPI into the SPB. However, specific nature of AFM measurements in contact mode should also be considered. The observed agglomeration of β_2-GPI on SPBs, as confirmed by our AFM experiments, was suggested as a reaction mechanism comprising two steps: i.) initial binding of the protein to the lipids, ii.) subsequent formation of protein clusters. Binding of the protein on the phospholipids was recognised to be mainly of electrostatic and also hydrophobic nature [14, 17]. The more negatively charged net surface of SPBs and less calcium, the greater binding of β_2-GPI to SPBs has been detected [17, 20]. When using discontinuous SPBs, β_2-GPI bound to mica patches when these are exposed. Because the mica surface is hydrophilic and slightly negatively charged, the interactions between protein and the support are likely to be mainly of electrostatic nature. Electrostatic interactions are, however, expected also in the interaction of antibodies and the mica surface. The orientation of IgG on mica is random. However, no binding of studied IgG to SPBs were observed.

Knowing the basic information about β_2-GPI and antibodies binding to mica and SPBs, crystallization of ANX A5 on SPB now had to be considered. It was found that the type of ANX A5 2-D crystal depends on the phosphatidylserine content in the phospholipid membrane and the calcium concentration [52-56]. In our study model with SPBs containing 30 % of PS and 10 mg/l of ANX A5 in HBS supplemented with 1.5 mM calcium, the p6 crystal form was predominately found. The vertical height of ANX A5 crystalline layer over phospholipids, measured with imaging, was determined to be approximately 2.6 nm and is in agreement with previous studies [47, 52-56].

When studying binding of β_2-GPI to SPBs in the presence of ANX A5, no binding or even desorption of the former from SPBs was observed. This effect could be explained by greater affinity of ANX A5 than β_2-GPI to SPBs under studied conditions (30 % L-α-PS, 10 mg/l ANX A5 in HBS supplemented with 1.5 mM calcium). The observed phenomena could also be explained by phase separation of acidic and neutral phospholipids in studied phospholipid bilayers, where ANX A5 concentrates negatively charged phospholipids, leaving less negatively charged phospholipids for β_2GPI binding, thus reducing the affinity of β_2GPI for binding to SPBs [57, 58]. When studying binding of antibodies in the presence of completely or incompletely crystallized ANX A5 on SPBs, no binding of antibodies to SPBs on their own was detected. Antibodies bound randomly to the exposed mica.

Knowing the background, our next step was joining all three reagents (β_2-GPI, antibodies and ANX A5) together in order to evaluate the pathological potential of anti-β_2-GPI through their effect on ANX A5 crystalline shield. When experimenting with isolated anti-β_2-GPI (sample anti-β_2-GPIA), the β_2-GPI-anti-β_2-GPI pair grew on SPBs, but only in the presence of incompletely crystallized ANX A5. No effect on crystalline ANX A5 was observed, when using the IgG fraction (which contained also anti-β_2-GPI) from the same patient A. The observed *in vitro* effect only by isolated anti-β_2-GPI could be explained by different titers of anti-β_2-GPI. Anti-β_2-GPI in sample anti-β_2-GPIA were 10 x more concentrated than in sample IgGA (100 x above the cut off, determined by an *in-house* anti-β_2-GPI enzyme-linked

immunosorbent assay [49]). Avidity and epitope specificity of anti-β_2-GPI for β_2-GPI in the samples IgGA and anti-β_2-GPIA were identical as a result of using the same polyclonal antibody fraction. However, patient A has shown (by an *in-house* kaotrophic anti-β_2-GPI enzyme-linked immunosorbent assay) high avidity anti-β_2-GPI profile, which should not be neglected, when considering the reasons for ability for growth of β_2-GPI-anti-β_2-GPI pair in the presence of incomplete crystalline ANX A5 on SPBs. Since antigen-antibody complex was unable to bind to SPB when the phospholipid surface was completely covered with ANX A5, we can deduce that the presence of pathological anti-β_2-GPI alone is not sufficient to cause disruption of ANX A5 crystalline shield, and therefore thrombosis. Our results correlate to clinical observations by which not all patients with positive aPL develop clinical manifestations and, in the ones that do, the thrombotic events occur only occasionally in spite of the persistent presence of aPL. Taken together, our results indicate that the hypothesis of aPL (more specifically, anti-β_2-GPI) mediated disruption of the ANX A5 protective shield is possible. However, the pathological potential of anti-β_2-GPI should be considered only as a necessary *"second hit"*. For thrombosis to develop, the *"first hit"* (endothelial, trophoblast cell damage, pathogenesis in ANX A5 synthesis or function) needs to be present as well [59, 60, 61].

To conclude, we confirmed the proposed thrombomodulatory effect of β_2-GPI- anti-β_2-GPI pair to ANX A5 by *in vitro* model with higher physiological similarity to circumstances in human body, and AFM ability to visualize the interaction in real time and at a nanomolecular scale. In addition, the visualization that the presence of β_2-GPI-anti-β_2-GPI pair itself is not sufficient to induce the pathological process is reported here for the first time.

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DECLARATION OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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