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Antiphospholipid antibody syndrome (APS) is an autoimmune thrombophilia mediated by autoantibodies directed against phospholipid-binding plasma proteins, mainly β 2 Glycoprotein I (β 2GPI)- a plasma apolipoprotein and prothrombin (PT). A subgroup of these antibodies termed "Lupus Anticoagulant" (LA) elongate in vitro the clotting times, this elongation not corrected by adding normal plasma in the detection system. The exact mechanism by which these autoantibodies induce thrombosis is not well understood. Resistance to natural anticoagulants such as protein C, impaired fibrinolysis, activation of endothelial cells to a pro-coagulant phenotype and activation of platelets, are among the mechanisms partially supported by experimental evidence. Artificially dimerized β 2GPI binds tightly to platelet membrane activating them. We search for mechanisms of natural dimerization of β 2GPI by proteins of the platelet membranes and found that platelet factor 4 (PF4) assembled in homotetramers binds two molecules of β 2GPI and this complex is recognized by anti- β 2GPI antibodies, the whole complexes being thrombogenic in terms of activating platelets as confirmed by p38MAP kinase phosphorylation and thromboxane B₂ production. Of note PF4/heparin complexes are also immunogenic triggering the production of anti-PF4/heparin antibodies which activate also platelets (the so-called "heparin-induced thrombocytopenia and thrombosis syndrome", HIT). The anti- β 2GPI antibodies activate platelets by their F(ab)₂, while the anti-PF4/heparin by their Fc fragments. Thus PF4 is a common denominator in the pathogenesis of APS and HIT which share also clinical characteristics such as thrombocytopenia and thrombosis.



A novel mechanism of thrombosis in antiphospholipid antibody syndrome

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ABSTRACT

Keywords:

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Antiphospholipid antibody syndrome (APS) is an autoimmune thrombophilia mediated by autoantibodies directed against phospholipid-binding plasma proteins, mainly β2 Glycoprotein I (β2GPI)-a plasma apolipoprotein and prothrombin (PT). A subgroup of these antibodies termed “Lupus Anticoagulant” (LA) elongate *in vitro* the clotting times, this elongation not corrected by adding normal plasma in the detection system. The exact mechanism by which these autoantibodies induce thrombosis is not well understood. Resistance to natural anticoagulants such as protein C, impaired fibrinolysis, activation of endothelial cells to a pro-coagulant phenotype and activation of platelets, are among the mechanisms partially supported by experimental evidence. Artificially dimerized β2GPI binds tightly to platelet membrane activating them. We search for mechanisms of natural dimerization of β2GPI by proteins of the platelet membranes and found that platelet factor 4 (PF4) assembled in homotetramers binds two molecules of β2GPI and this complex is recognized by anti-β2GPI antibodies, the whole complexes being thrombogenic in terms of activating platelets as confirmed by p38MAP kinase phosphorylation and thromboxane B2 production. Of note PF4/heparin complexes are also immunogenic triggering the production of anti-PF4/heparin antibodies which activate also platelets (the so-called “heparin-induced thrombocytopenia and thrombosis syndrome”, HITT). The anti-β2GPI antibodies activate platelets by their F(ab)2, while the anti-PF4/heparin by their Fc fragments. Thus PF4 is a common denominator in the pathogenesis of APS and HITT which share also clinical characteristics such as thrombocytopenia and thrombosis.

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1. Introduction

Antiphospholipid Antibody Syndrome (APS) is an autoantibody-mediated acquired thrombophilia characterized by recurrent arterial or venous thromboses and/or pregnancy morbidity, accompanied by antiphospholipid (aPL) antibodies [1]. Although aPL antibodies were originally thought to recognize negatively charged phospholipids (PLs), it became evident that they recognize in fact phospholipid-binding plasma proteins, mainly a plasma apolipoprotein known as β2-glycoprotein I (β2GPI) [2] and prothrombin [3]. A subgroup of these antibodies termed “Lupus Anticoagulant” (LA) elongate *in vitro* the clotting times, this elongation not corrected by adding normal plasma in the detection system [4]. A brief history of the above discoveries gives an idea of the exhaustive scientific efforts to unravel the puzzling nature of these antibodies and their cause-and-effect relationship to thrombosis.

2. Historical background

In 1907 Wasserman developed a serologic test for syphilis using as antigen PLs from saline liver extracts of fetuses with congenital syphilis [5]. In 1941 Pangborn demonstrated that the antigen in the above serologic test for syphilis was an anionic PL which she named ‘cardiolipin’ because it was isolated from bovine heart [6]. Moore and Mohr in 1952 described that patients with systemic lupus erythematosus (SLE) had persistently positive serologic test for syphilis in the absence of infection [7]. At the same time, Conley and Hartmann described an acquired circulating inhibitor of coagulation *in vitro*, in two patients with SLE who had no bleeding tendency [8]. On the contrary, Bowie et al. in 1963 described the association of this anti-coagulant factor with thrombosis [9] and ten years later Feinstein and Rapaport used the term LA for this *in vitro* coagulation inhibitor [10]. However LA proved to be a misnomer because it induces thrombosis *in vivo* and also occurs in a variety of conditions unrelated to SLE, in other autoimmune disorders, and in patients receiving drugs such as procainamide and chlorpromazine, in children with recent acute viral infections, in patients with HIV infection and in patients with unexplained

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venous or arterial thrombosis [1]. Subsequently, chronic false positive tests for syphilis were associated with arterial thrombosis and thrombocytopenia [11], while LA and aPL were associated with recurrent abortions [12]. In 1985, an enzyme linked immunosorbent assay (ELISA) was described for the semi-quantitative detection of aPL antibodies using CL as the antigen (aCL antibodies) [13]. These antibodies were associated with thrombocytopenia, recurrent abortions and recurrent thrombotic events leading to the description of the so-called “antiphospholipid antibody syndrome” [14]. Subsequent research has revealed that LA and aCL comprise different antibody subgroups and that plasma can be separated into fractions containing these two separate activities [15]. It was also shown that β 2GPI was important for the binding of aCL antibodies to PLs and in fact antibodies recognized β 2GPI bound to the PL surface [2]. Initially, β 2GPI was considered as a serum co-factor for the binding of aCL antibodies to PLs but later it was shown that β 2GPI could bind directly to γ -irradiated polystyrene plates and be recognized by aPL antibodies in ELISA, in the absence of PLs, because irradiation generates negative charges on the polystyrene surface which are important for the binding of β 2GPI. The main antigenic target of aPL is β 2GPI that binds to anionic PLs; this is a 50 kDa plasma protein composed of five complement control protein modules, which are termed domains I through to V. β 2GPI has a lysine-rich domain in the C terminus region of domain V, responsible for the binding to anionic PLs but lacks any intracellular domain. At the same time it was shown that LAs constitute a heterogeneous group of antibodies directed also against PL binding proteins most often β 2GPI [16] and prothrombin [3].

3. Antiphospholipid antibodies activate coagulation, induce a pro-coagulant phenotype of cells and inhibit fibrinolysis *in vivo*

The dichotomy between the *in vitro* anti-coagulant effect and the *in vivo* pro-coagulant activity of aPL antibodies was a mystery until the discovery of the anti-prothrombin specificity characterizing at least subgroups of these antibodies. Then, the elongation of activated partial thromboplastin time (aPTT) should reflect probably the disruption of the prothrombinase complex, that is the complex of activated coagulation factors X and V (Xa and Va respectively) along with prothrombin which bind en bloc to PL surfaces. The *in vivo* pro-coagulant activity of aPL antibodies remains still a process not well understood. Resistance to natural anticoagulants, impairment of fibrinolysis, activation of pro-coagulant phenotype of endothelial cells and platelets, activation of the coagulation cascade have been proposed to explain the pro-coagulant activity of these antibodies.

3.1. Resistance to natural anticoagulants

Activated protein C (APC) binds the pro-coagulant factors Va and VIIIa, inactivating them. Anti- β 2GPI/ β 2GPI complexes inhibit the APC activity *in vivo*; it seems that anti- β 2GPI/ β 2GPI probably prevent APC/Va/VIIIa complexes for binding to PLs, or even disrupt these complexes [17] (Fig. 1). In patients with LA, degradation of factor Va was decreased and failed to improve with the addition of exogenous APC. Similar results were obtained with purified IgG from patients with LA and/or aCL [18]. It was suggested that this phenomenon could be attributed to antibodies against complexes of PLs with plasma proteins, including protein C. However the mechanism by which anti- β 2GPI and/or LA antibodies induce APC resistance is not entirely clear. Conflicting data between studies and lack of large scale population and controlled studies do not support the concept that APC resistance may be a general mechanism of thrombosis in APS.

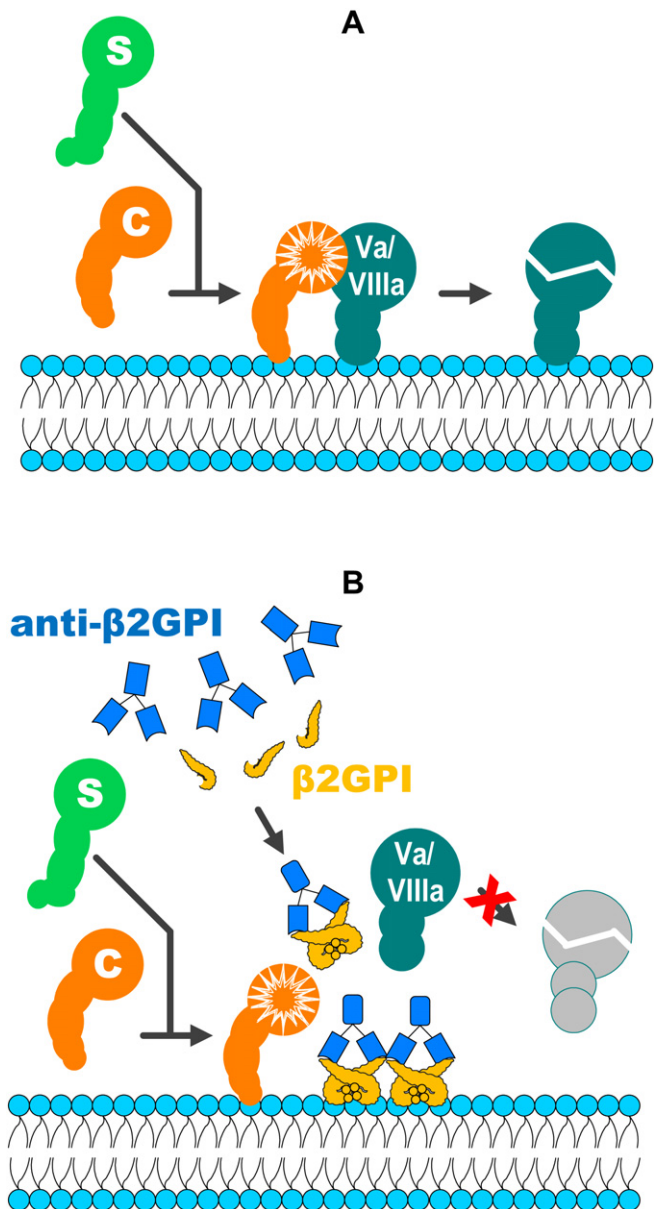


Fig. 1. A. Protein S activates protein C, which in turn binds the pro-coagulant factors Va and VIIIa, inactivating them. B. Anti- β 2GPI/ β 2GPI complexes prevent APC/Va/VIIIa complexes for binding to phospholipids or even prevent the formation of these complexes.

3.2. Impaired fibrinolysis

The fibrinolytic system in mammals consists of an inactive proenzyme, plasminogen, which is converted to the active enzyme, plasmin; this, in turn, degrades fibrin into fibrin degradation products. The conversion of plasminogen to plasmin is enhanced by two immunologically distinct, physiologic plasminogen activators: tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Inhibition of the fibrinolytic system may occur at the level of the plasminogen activators by specific plasminogen activator inhibitors (PAI-1 and PAI-2) or at the level of plasmin, by α 2-antiplasmin [1].

Monoclonal anti- β 2GPI significantly suppressed the intrinsic fibrinolytic activity *in vitro*. The activity of intrinsic fibrinolysis was calculated by subtracting plasmin activity in the presence of kaolin [19].

Monoclonal aCL appear to inhibit fibrinolysis by a β 2GPI dependent increase in PAI-1 activity [20].

Another important development in the research concerning the relationship of APS with impaired fibrinolysis is the recent discovery of high affinity antiplasmin antibodies in patients with APS; some of these inhibited plasmin [21]. Another group that studied the anti-fibrinolytic effect of IgG from APS patients showed that IgG significantly retard fibrin dissolution by plasmin [22]. The above results demonstrate that there are two kinds of aCL that may inhibit fibrinolysis: the former suppresses plasmin generation by elevating PAI-1 activity, and the latter directly inhibits plasmin activity.

Finally, a possible role of lipoprotein (a) [Lp(a)] in fibrinolytic defects of patients with APS was addressed in a 1998 study. Plasma levels of Lp(a) were found to be significantly elevated in patients with APS. Furthermore, patients with maximal elevation of Lp(a) showed a diminished fibrinolytic activity, estimated by lower D-dimer and higher PAI levels [23].

3.3. Endothelial cell activation to a pro-coagulant phenotype

Since APC resistance measurements were based on *in vitro* assays and the presence of aPL may interfere with these assays, intensive research was undertaken to study the interaction of aPL with endothelial cells and platelets, with the hope that any evidence should reflect better the *in vivo* situation. Human umbilical vein endothelial cells (HUVECs) were used in mono-layers and incubated with various types of aPL antibodies, such as LA, anti- β 2GPI, or even crude serum from APS patients. Affinity-purified anti- β 2GPI bound more effectively to HUVEC prior to fixation of the cells onto ELISA plates (cell ELISA) in the presence of purified β 2GPI in a dose-dependent manner. In addition HUVECs expressed on their surface adhesion molecules, such as the endothelial leukocyte adhesion molecule-1 (ELAM-1) [24]. The upregulation of adhesion molecules, the secretion of proinflammatory cytokines, and the modulation of arachidonic acid metabolism were taken as the indicators of HUVEC activation [25].

The ability of patient derived aCL antibodies to activate endothelial cells was also evaluated using the so-called “pinch induced thrombosis model”. This model involves injection of 500 μ g of IgG aPL preparation initially and 48 h later in CD1mice. Subsequently, the right femoral vein is pinched with a pressure of 1500 g/mm² to induce a standardized thrombogenic injury. Clot formation and dissolution in the transilluminated vein are monitored with a microscope equipped with a closed circuit video system. With this model, it was shown that about two thirds of the patient-derived monoclonal antibodies were thrombogenic, and some of them caused *in vivo* leukocyte adhesion to ECs in the microcirculation and enhanced expression of vascular cell adhesion molecule-1 (VCAM-1) as compared to that induced by the H2 control human monoclonal IgG [26]. Monocyte chemoattractant protein I (MCP1) in HUVEC as well as MCP1 RNA expression were increased in the presence of aPL, which suggested that this was a mechanism for adhesion of mononuclear cells to the endothelium [27]. The interaction between β 2GPI and Glu-plasminogen was recently reported and suggested that this may play an important role in increasing the generation of plasmin in endothelial cells; consequently, interference on these endothelial cells by anti- β 2GPI would generate thrombosis through an increase of intracellular adhesion molecule-1 (ICAM-1) and a decrease in the expression of thrombomodulin [28].

3.4. Endothelial cell activation by aPL resembles activation of endothelium by components of innate immunity

Incubation of HUVEC with aPL resulted in a re-distribution of nuclear factor κ B (NF κ B) from the cytoplasm to the nucleus. Over

several hours, this was accompanied by increased expression of tissue factor (TF) and of the leukocyte adhesion molecules ICAM-1, VCAM-1, and E-selectin. Inhibition of the nuclear translocation of NF κ B abolished the above response. Incubation of HUVEC with TNF instead of aPL resulted in a more rapid response in the same direction [29]. The above data indicated that aPL may activate natural immunity by interacting with toll-like receptors. Indeed, anti- β 2GPI reacting with β 2GPI on the EC surface activated the toll-like receptor/IL-1 receptor family of proteins [30]. In that respect, aPL are as powerful as TNF in inducing NF κ B activation and E-selectin expression [31]. Another mechanism for abrogating the anti-coagulant activities of endothelial cells was disruption of annexin A5 by aPL [32]. It seems that priming of endothelial cells as detected by the expression of phosphatidylserine on the cell surfaces is important for their activation by aPL antibodies [33]. Low concentrations of cytokines such as TNF or IL-1 may play a role in priming [34].

Crystallization experiments have shown that β 2GPI binds by domain V to PL surfaces of endothelial cells but it lacks any intracellular domain [35]. Therefore, it is difficult to understand how cross-linking of β 2GPI by anti- β 2GPI can be an adequate mechanism for signaling. Furthermore, recent evidence shows that heparin, which is used in culture media for HUVEC, binds β 2GPI and makes it available for proteolytic cleavage by plasmin in its fifth domain [36]. The cleaved β 2GPI is not capable of binding to EC surfaces. This means that, in culture conditions, the stoichiometry of the interaction between anti- β 2GPI and β 2GPI is uncertain. As a result, the experiments designed using culture media with HUVEC have to be considered with caution since they are not always reproducible. Besides not all authors describe so clearly activated HUVEC after their interaction with aPL [37].

We searched for homologies between β 2GPI and proteins that belong to the TNF receptor family, based on the hypothesis that anti- β 2GPI recognize such proteins in case they possessed structural homology with β 2GPI; an additional reason for this approach was the evidence that activation of endothelial cells by aPL required signals delivered by the TNF receptor family of proteins. A protein sequence database search revealed a homology between amino acid residues 7–13 of β 2GPI and 239–245 of CD40. Antibodies against this CD40 peptide detected in patients with APS were isolated and were shown to react against β 2GPI. On the other hand, anti- β 2GPI were also isolated from the sera of the patients using a combination of affinity and ion exchange chromatography and were shown to recognize the CD40 peptide [1]. The above findings are in line with previous findings that suggest that a combination of CD40Ligand with IL-4 was enough for activation of endothelial cells in a manner similar to endothelial cell activation by TNF alone [38]. A significant increase of the expression of TF, IL-6, IL-8, and inducible nitric oxide synthetase in HUVEC cultured in the presence of aPL from patients with APS, was detected, while all these processes involved phosphorylation of p38 mitogen associated protein kinase (MAPK) and activation of NF κ B [39].

3.5. Platelet activation

Platelet preparations can be obtained in the absence of heparin, contrary to what happens with endothelial cells. Therefore, the interaction of β 2GPI with platelet surfaces cannot be blocked by heparin [36]. Furthermore platelets provide a surface for coagulation reactions, and they have multiple receptors that can interact with antibodies; thus, platelets constitute an important biological material to study the mechanisms implicated in thrombosis in APS. Increased urinary excretion of the platelet-derived thromboxane B2 (TXB2) and a significant reduction of the vascular prostacyclin (PGI2) in patients with LA and aPL, provides an indirect evidence for

platelet activation in APS. In addition the F(ab')₂ fractions of aPL antibodies derived from APS patients increased aggregation and serotonin release of platelets stimulated by low doses of thrombin.

Chimeric dimers of β 2GPI were constructed by making fusion proteins of β 2GPI with the aPL4 component of coagulation factor IX. These proteins undergo dimerization spontaneously and when incubated with platelets primed with low doses of thrombin they increase platelet adhesion and thrombus formation in a flow system. In another set of experiments, the same group, using a co-immunoprecipitation assay, demonstrated that a splice variant of apolipoprotein receptor (apoER2') in the platelet membrane interacted with dimers of β 2GPI and mediated platelet activation [40]. The receptor apoER2' was identified by using monoclonal antibodies to it and was detected by Western blotting. The idea behind searching for apoER2', as a possible ligand of β 2GPI was that this is an apolipoprotein receptor, unique in platelets.

The GPIIb/IIIa subunit of the GPIIb/IX/V receptor has also been shown to bind β 2GPI and to induce TXB₂ production *in vitro*. Searching for interaction of the GPIIb/IX/V receptor with β 2GPI was based on the knowledge that this receptor "has many ligands" and proving of this interaction was carried out by direct binding assays [41].

The intracellular events involved in platelet activation are not fully understood. However platelets primed with low doses of thrombin and subsequently exposed to affinity-purified IgG or IgG F(ab)₁ fragments from sera of patients with APS, a significant increase in phosphorylation of p38 MAPK and of cytosolic phospholipase A₂ (cPLA₂) but not of ERK 1/2 MAPK, was observed. In addition, platelets treated with F(ab)₁2 and thrombin produced significantly larger amounts of TXB₂ [39].

Identification of apoER2' and GPIIb/IX/V receptors as potential adaptor proteins of β 2GPI on platelet surface, was carried out by very elegant experiments and was further supported by functional studies indicating an activated platelet phenotype after interaction with anti- β 2GPI/ β 2GPI complexes. Interestingly, signaling patterns through the above receptors are almost identical to those operating after interaction of platelet surfaces with anti- β 2GPI [39]. However, there is not analytical work behind this identification and the whole process was based mainly on "fishing expedition".

4. A nil hypothesis: proteins derived from platelet membrane extracts, which bind to a β 2GPI affinity column, are those that potentially interact with β 2GPI *in vivo*

4.1. β 2GPI binds platelet factor 4 (PF4)

Using novel protocols based on ultracentrifuges in a discontinuous sorbitol gradient we purified platelet membranes obtained by platelet apheresis from patients with APS as well as normal individuals. Platelet membrane proteins were extracted and passed through a β 2GPI affinity column. It was shown that the only protein which was constitutively eluted from the β 2GPI affinity column loaded either with platelet membrane proteins both from normal subjects and APS patients was Platelet factor 4 (PF4), a protein derived from platelet α -granules [42]. PF4 is a member of the C-X-C chemokine family with a molecular mass of approximately 7800 Da [43]. PF4 is secreted by activated platelets but has also the ability to bind to the platelet surface [43]. PF4 has high affinity ($K_D = 5\text{--}20$ nM) for heparin and other anionic glycosaminoglycans (e.g., endothelial cell surface or platelet surface GAGs) [44]. Identification of PF4 was based on Nano-high performance liquid chromatography/mass spectrometry (Nano-HPLC/MS) analysis of proteins eluted from the column after their separation through SDS-PAGE on a 12% gel [42]. The interaction of β 2GPI with PF4 was further supported by direct binding assays, both in solid phase (biotinylated PF4 bound to immobilized β 2GPI on ELISA plates in a dose-dependent fashion) or

in solution (biotinylated β 2GPI in the presence of PF4 both in solution was subjected to precipitation by binding to avidin-agarose beads and the precipitant was shown to contain PF4). Complexes of PF4 with β 2GPI were more effectively than β 2GPI alone recognized by APS sera in ELISA [42]. Interestingly, more than a decade ago, efforts to purify β 2GPI were partially successful, since a "mysterious" 8 kDa protein was co-present in the β 2GPI preparations and disappeared on treatment with SDS or urea [45].

4.2. β 2GPI polymorphisms may be important for interaction of β 2GPI with other proteins and predict patients with APS

The above findings may encourage another way of explaining the association of β 2GPI polymorphisms with APS. It is possible that β 2GPI may interact with other proteins to make immunogenic as well as thrombogenic complexes and this is dependent on slight differences of the molecule due to the polymorphism of β 2GPI gene.

Four alleles of the β 2-GPI gene have been identified by isoelectric focusing termed ApoH*1, ApoH*2, ApoH*3 and ApoH*4, the latter being present only in black people [46]. ApoH*3 can further be divided into two subtypes, ApoH*3w and ApoH*3B, based on different monoclonal antibody recognition. These alleles derive from the genetic variation in β 2-GPI DNA sequences. At the DNA level 8 single nucleotide polymorphisms (SNPs) have been found within the coding region [47]. The major SNPs are located at codons 88 (exon 3), 135 (exon5), 247 and 306 (exon 7) and 316 (exon 8). The SNP at codon 88 (Ser \rightarrow Asn) was reported to be present in all ApoH*1 allele carriers while the SNP at codon 316 (Trp \rightarrow Ser) is present in all ApoH*3w carriers.

The most important SNP, which is correlated with anti- β 2-GPI antibody production in patients with primary APS, is located within domain V of the β 2-GPI protein and regards the substitution at position 247 of Val to Leu. Val247 itself may be a genetic risk factor for development of APS and could be important in the formation of β 2-GPI antigenicity [48] as it has been shown by several studies:

- (i) In pediatric patients, an association has been demonstrated for the Val247Leu substitution and both the development of aPL, and aPL clinical manifestations [49].
- (ii) In Mexican subjects, the Val347 was found to be in significantly higher frequencies in anti- β 2-GPI-positive patients and in patients with arterial thrombosis than in anti- β 2-GPI-negative patients or donors [50].
- (iii) In Japanese patients, V247 beta2-GPI allele was associated with increased levels of beta-thromboglobulin and platelet factor 4 as well as with for the development of cerebral infarction through platelet activation [51]. In addition, the Val247 β 2GPI allele was associated with both a high frequency of anti- β 2GPI antibodies and stronger reactivity with anti- β 2GPI antibodies compared with the Leu247 β 2GPI allele [48].
- (iv) In Brazilian subjects the V247-encoding allele was found in significantly higher frequency patients with APS than in normals and was associated with arterial and venous thrombosis and the presence of anti- β 2GPI antibodies [52].

4.3. Interaction of PF4 tetramers with β 2GPI induces β 2GPI dimerization

The crystal structures of human β 2GPI and PF4 tetramer were imported ICM-Pro software (Molsoft Inc). To investigate the possible orientation of PF4 tetramer on the β 2GPI surface, docking simulations were performed. Four different properties were taken

into account (van der Waals, electrostatic, hydrogen bonding, and hydrophobic potential) [53].

Docking solutions suggested that PF4 binds to negatively charged patches of domains III to V in the inner curve of β 2GPI (Fig. 2). In this regard, the positively charged surface of PF4 tetramer interacts with the negatively charged regions of β 2GPI domains III to V. Overall, the interaction interface in β 2GPI is formed mainly by residues Arg135, Val136, Arg148, Thr150, Val152, and Gly163 of domain III; residues Pro224, Glu225, Glu226, Glu228, and Ser236 of domain IV; and residues Gln257, Gly258, and Glu259 of domain V. In PF4, the interaction area includes residues Leu8,

Val13, Lys14, and Gln56 of chain A and residues Lys14, Ser17, Arg22, Lys66, and Glu69 of chain C of the tetramer. Notably, no residues of chains B and D of the tetramer are used for the binding to β 2GPI. Therefore, the residues of chains B and D that are homologous to the residues involved in PF4– β 2GPI interaction are free for binding to another β 2GPI molecule, allowing its dimerization. As shown in Fig. 2, this PF4-mediated dimerization of β 2GPI can occur without any steric clashes. On the tripartite complex formation the high antigenic regions of domain I are placed in a way that allows their recognition by antibodies (Fig. 2). The oligosaccharides of β 2GPI do not seem to inhibit interaction with PF4 [42].

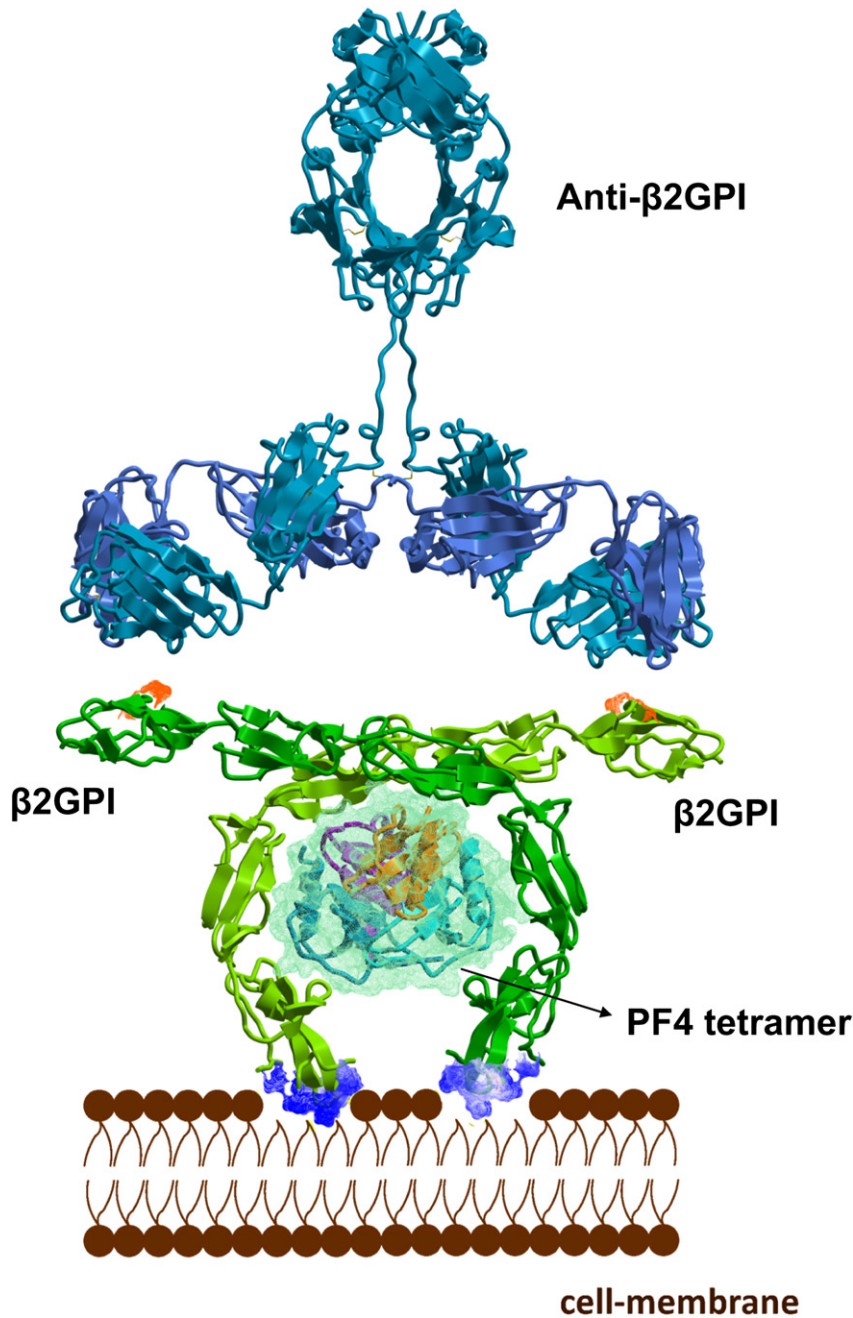


Fig. 2. Model for the mode of recognition of β 2GPI antigen by autoantibodies. Platelet factor 4 tetramer binds to β 2GPI, promoting the natural dimerization of the latter. Upon dimerization of β 2GPI, the epitopes—that thrombosis-associated antibodies recognize on domain I—are arranged in a geometry that precisely fits to the 2 antigen-binding sites of an antibody, allowing their bivalent recognition by low-affinity autoantibodies. On the opposite site of the β 2GPI–PF4 complex, positively charged patches in domain V of β 2GPI are involved in interaction with negatively charged membranes.

4.4. Anti-β2GPI antibodies recognize complexes of PF4 tetramers with dimerized βGPI, but also force these molecules to form such complexes

Size exclusion chromatography using recombinant PF4, β2GPI and purified anti-β2GPI revealed that a) PF4 tends to be eluted in tetramers, b) PF4 tetramers bind a unique molecule of β2GPI, but c) when anti-β2GPI was also added in the column, one molecule of this antibody bound to a molecular complex of PF4 tetramer with 2 molecules of β2GPI [(PF4)₄-(β2GPI)₂]. Therefore anti-β2GPI antibodies not only bind to (PF4)₄-(β2GPI)₂ complexes, but also force these molecules to make these complexes [42].

Complexes of the type of (PF4)₄-(β2GPI)₂ activate platelets and this activation was maximized after their incubation also with anti-β2GPI, as shown by TXB2 production and p38 MAPK phosphorylation [42].

5. Implications of PF4/β2GPI interaction for the pathogenesis of APS

PF4 has a proven pro-coagulant role: (1) inhibition of heparin dependent acceleration of thrombin inactivation by antithrombin [54], (2) potentiation of platelet aggregation [43], (3) aggregation defect of platelets in a PF4 knockout mice in response to low doses of thrombin [55], and (4) impaired platelet thrombus formation that was corrected by infusing PF4 [55]. PF4 however enhances generation of activated protein C *in vitro* and *in vivo* expressing also anti-coagulant action [56]. PF4–heparin complex formation is highly immunogenic and induces anti-PF4–heparin antibodies in a minority of heparin-treated patients. In the presence of these autoantibodies, the complexes activate platelets mainly through FcγRIIa receptors, [57]. As a result a syndrome characterized by thrombocytopenia and thrombosis,

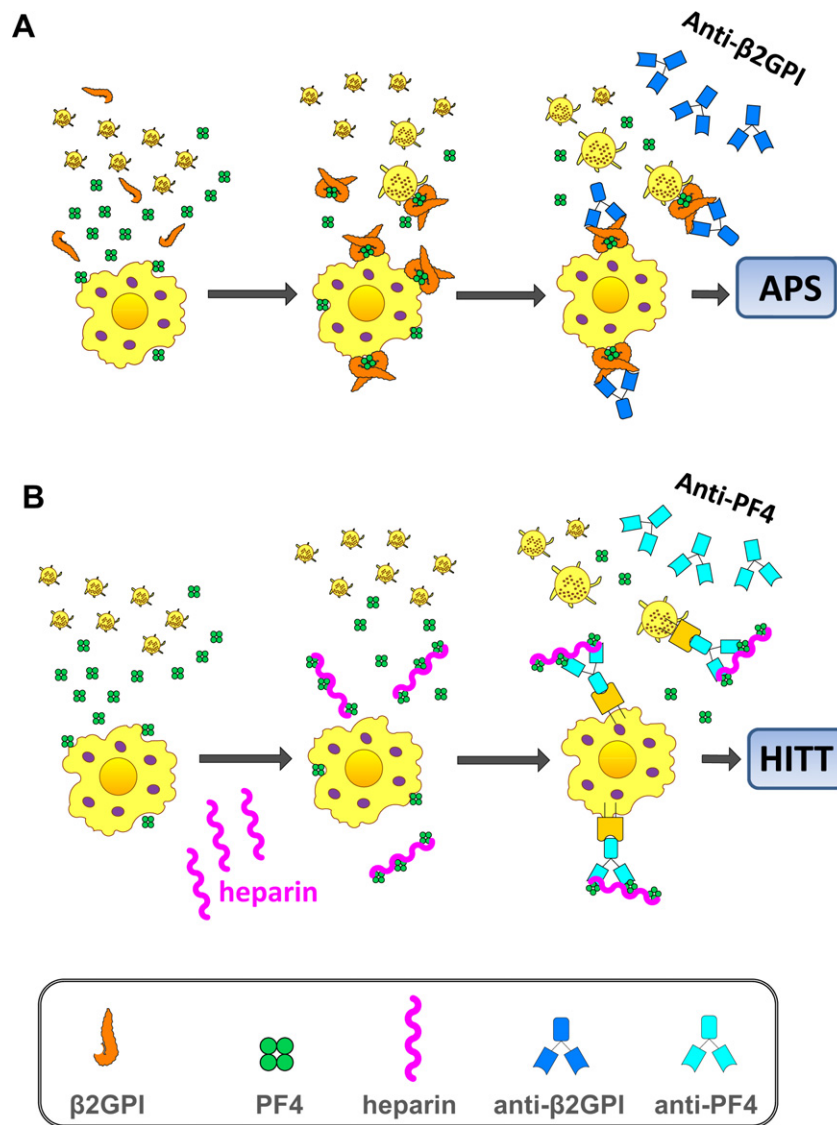


Fig. 3. Mechanistic basis of APS and HIT: Binding of both anti-β2-GPI and anti-PF4 autoantibodies to PF4/β2-GPI – containing complexes generate pathologic immune complexes, with 2 distinct mechanisms of cellular activation, one via the F(ab)₂ part of the β2-GPI-IgG (in APS) and one via the Fc region of the anti-PF4-IgG antibodies (in HIT). (A) Platelets are activated and release a great deal of PF4. PF4 is complexed with dimmers of serum β2GPI and the complexes are attached on the surface of both the platelets and monocytes. These complexes are antigenic targets for anti-β2GPI antibodies on platelets, monocytes and endothelium. In this regard, anti-β2GPI antibodies bind and activate these cells, leading to the production of pro-coagulant molecules such as tissue factor. (B) Following heparinization, PF4 becomes complexed to the circulating heparin. The circulating free PF4/heparin induces antibody production, and since there remain antigenic targets on platelets and monocytes, the antibodies bind to these cells and activate them, resulting in both the thrombocytopenia and prothrombotic state seen in HIT.

the so-called “heparin-induced thrombocytopenia and thrombosis (HITT)” is developed. PF4 seems to be a common denominator in both syndromes, APS and HITT, which share similar clinical manifestations such as thrombocytopenia and thrombosis (Fig. 3). We however blocked the FcγRIIa receptor and the activation of platelets in the presence of anti-β2GPI [(PF4)₄-(β2GPI)₂] complexes was still present implying that anti-β2GPI in APS operate via their F(ab)₂ fragments (Fig. 3).

6. Platelet factor 4 expression in non-PLT cells

Platelet factor 4 (PF4) is a CXC tetrameric, cationic chemokine synthesized by megakaryocytes and stored in platelet α-granules, where it constitutes 25% of the protein. It is also found bound to the luminal vascular endothelial surface. Moreover PF4 can induce phagocytosis and the generation of reactive oxygen metabolites [58] and is also able to increase the expression of E-selectin by endothelial cells [59]. Although platelets represent the primary source of PF4, the protein is expressed at lower levels in other cells of the immune system including cultured T cells, monocytes, and endothelial and smooth muscle cells. These levels, however, can be significantly increased upon cell stimulation. In this regard, PMA (phorbol 12-myristate 13 acetate) stimulates human T cells immediately to release high amounts of PF4 [59]. Similarly, PF4 expression is upregulated in monocytes by thrombin via proteinase-activated receptors [60]. In addition, PF4 is significantly upregulated in dendritic cells in patients after multiple trauma [61]. It is known that dendritic cells (DCs) express proinflammatory transcriptomic products early after trauma and therefore they produce PF4 and their PF4 concentration is significantly correlated with the severity of the injury. Upon its release, PF4 exhibits immuno modulatory effects, such as monocytic chemotaxis and prevention of monocyte apoptosis. The above finding may explain the association of severe trauma with the catastrophic form of the syndrome described previously [62]. We are very pleased to contribute this paper which presents a novel mechanism of thrombosis in the antiphospholipid antibody syndrome to honor Dr. Harry Moutsopoulos in this distinguished series on auto-immunologists [63–66].

7. Concluding remarks

A novel mechanism of thrombosis induced via the action of anti-β2GPI is described: This action is mediated by dimerization of β2GPI by PF4 tetramers; dimerization takes place mainly in the presence of anti-β2GPI antibodies which in turn bind to the PF4/β2GPI complexes and activate platelets. Almost every cell type can be a source of PF4 especially under stimulation by various stimuli. Since PF4 and β2GPI occur in abundance in plasma the preformed PF4/β2GPI complexes may prime several kinds of cells such as platelets, endothelial cells and monocytes to co-ordinate their actions towards coagulation. At the same time PF4 expression by dendritic cells makes possible that PF4/β2GPI complexes may activate immune receptors on B- and T-cells in the lymph nodes and trigger the production of anti-β2GPI antibodies.

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