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Keywords
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Beta-2 glycoprotein I and its role in antiphospholipid syndrome—lessons from knockout mice

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Abstract

The antiphospholipid syndrome is characterized by the presence in serum of autoantibodies against \( \beta_2 \)GPI. Although the role of \( \beta_2 \)GPI in the pathogenesis of antiphospholipid antibody syndrome (APS) is well recognized, its exact physiological functions still remain undisclosed. Several interactions of \( \beta_2 \)GPI with components of the coagulation cascade have been proposed, resulting in both procoagulant and anticoagulant effects. Additionally, \( \beta_2 \)GPI has been implicated in the mechanism of recurrent fetal loss entailed in APS. Recently, using a homologous recombination approach, reproduction of mice homozygous for deletion of the \( \beta_2 \)GPI gene has been feasible. \( \beta_2 \)GPI knockout mice offer a valuable tool for revealing the physiological role of the protein. These mice show decreased in vitro ability for thrombin generation. Furthermore, although mice lacking \( \beta_2 \)GPI are fertile, the success of early pregnancy is moderately compromised and functional \( \beta_2 \)GPI is believed necessary for optimal implantation and placental morphogenesis.

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Keywords: Antiphospholipid syndrome; \( \beta_2 \) glycoprotein I; Autoantibodies; Transgenic or knockout; Mice; Thrombin; Pregnancy loss

Introduction

The antiphospholipid antibody syndrome (APS) is determined by a constellation of clinical features (mainly recurrent venous and arterial thrombosis, recurrent fetal loss, and livedo reticularis) in combination with the detection of antiphospholipid antibodies (aPL) in the sera of affected patients [1,2]. The disorder may occur alone (referred as the primary APS) or in association with systemic lupus erythematosus (SLE), other autoimmune diseases, and rarely with other diseases, certain infections, and drugs (secondary APS) [2–5]. The aPL can be detected either via the lupus anticoagulant (LA) in vitro bioassay or via commercially available ELISA assays for antibodies interacting with cardiolipin and/or \( \beta_2 \) glycoprotein-I (\( \beta_2 \)GPI).

The plasma protein \( \beta_2 \)GPI is a phospholipid binding protein with anticoagulant properties. \( \beta_2 \)GPI constitutes the major antigenic target for aPL circulating in the serum of APS patients [6]; therefore, the term “antiphospholipid antibodies” is actually a misnomer. It is currently evident that the \( \beta_2 \)GPI ELISA has greater specificity and positive predictive value than the cardiolipin ELISA for the diagnosis of clinically significant APS [7,8]. These data confirmed previous experimental results on the role of \( \beta_2 \)GPI-dependent aPL in distinguishing between APS occurring in the setting of autoimmune diseases and the transient occurrence of aPL associated with infections [9]. In addition, it has been shown that most cases of clinically significant LA are associated with concomitant presence of anti-\( \beta_2 \)GPI antibodies in the sera of APS patients [10,11]. Taken together, these data argue for a key role of \( \beta_2 \)GPI in the molecular pathophysiology of APS (summarized in Fig. 1).

\( \beta_2 \) glycoprotein-I

Protein chemistry

\( \beta_2 \)GPI is a single chain glycoprotein, with a mass of approximately 54 kDa [12]. Its 326 amino acid sequence consists of five structurally similar short consensus repeat (SCR) domains [13]. This characterizes \( \beta_2 \)GPI as a member
of the complement control protein family [14]. The first four domains are able to form disulfide bridges, forming a “looped-back” configuration (known also as a sushi domain) [15]. The domains I–II are of unknown function and project away from the phospholipid surface into the plasma space [16]. The central portion (domains III and IV) is heavily glycosylated, separating the phospholipid binding site (domain V) from domains I–II [16,17]. Both domains I and IV have been suggested to contain the major epitopic region for most anti-β2GPI autoantibodies [18,19].

The fifth domain of β2GPI contains a positively charged (lysine-rich) region and also a hydrophobic loop terminal area predicted to be surface exposed [16,17]. There is ample evidence that both these regions on domain V mediate the phospholipid binding properties of β2GPI: Site-directed mutagenesis of Lys residues in the lysine-rich region (amino acids 281–288) abolishes the binding of β2GPI to CL, inhibiting also the binding of antibodies to β2GPI in a CL ELISA [20]. Likewise, cleavage by both plasmin and (less effectively) factor Xa has been demonstrated to occur at the terminal region of domain V (Lys 317-Thr 318) [21,22]. This cleavage has been also predicted by modeling to affect the position of the lysine-rich region, which is considered as the main phospholipid binding region of β2GPI [20] (Fig. 1). Not surprisingly, therefore, the cleaved β2GPI shows reduced ability to bind phospholipids in vivo [21,22]. Additionally, genetic polymorphisms resulting in alterations of the 20 residue tail disrupt the integrity of configuration of domain V, thus limiting the ability of β2GPI to bind negatively charged molecules such as phosphatidylserine [23]. Recently, it was shown that heparin binds to the lysine-rich site located within the fifth domain of β2GPI [24]. Moreover, heparin at therapeutic concentrations is able to enhance the plasmin-mediated cleavage of the C-terminal region (Lys 317-Thr 318) of β2GPI [24]. These data indicate the importance of the fifth domain of β2GPI for binding to anionic phospholipids and heparin.

β2GPI is one of the most abundant proteins in human serum, with a mean serum level of 200 mg/L, second only to fibrinogen among the plasma proteins involved in clotting [25]. There is a wide range of interindividual variation in β2GPI plasma levels, arising from nucleotide polymorphisms of the β2GPI gene [26,27] that maps to chromosome arm 17q [13]. The protein exists in the circulation predominantly in the free form, but also in lipid-bound form, and satisfies the criteria for classification as an apolipoprotein [28]; hence, it has also been termed apolipoprotein H [29]. β2GPI is synthesized mainly in the liver [13] and also in intestinal epithelial cells [30] and placenta cells [31]. β2GPI in plasma is able to bind to anionic or zwitterionic phospholipid without calcium ions. The precise function of the protein in plasma is still unresolved, although β2GPI in vitro binds to anionic phospholipids, producing an apparent anticoagulant effect by occupying the procoagulant binding sites [32]. Interestingly, individuals without detectable β2GPI have been identified who appear clinically well [33,34].

β2GPI and coagulation factors

A number of possible in vitro functions of β2GPI for the control of intravascular clot formation have been proposed. These include both procoagulant [35,36] and anticoagulant activity [37,38] at different stages of the coagulation cascade. Similarly, data on the role of antibodies against β2GPI are incongruous [39,40]. Interestingly, β2GPI-deficient individuals are apparently not at risk of thrombosis [34] and most of their hemostatic and fibrinolytic markers are normal [41].

It seems therefore that the role of β2GPI in APS-related thrombosis is rather more qualitative than quantitative, with aPL possibly interfering with the physiological functions of β2GPI, including the protein’s interactions with coagulation factors. β2GPI normally inhibits the generation of factor Xa in the presence of activated platelets, and the presence of antiphospholipid antibodies results in protracted, unopposed factor Xa generation [38]. On the other hand, plasmin and factor Xa can cleave the positively charged sequence Lys 317-Thr 318 at the fifth domain of β2GPI, impairing the ability of β2GPI to bind phospholipids [21,22]. Moreover, heparin binding to the fifth domain of β2GPI at concentrations achieved in vivo, greatly enhances the plasmin-mediated cleavage of β2GPI [24].

Association of antibodies against β2GPI with protein C activity has been reported by different groups [40,42,43]; however, a direct interaction between β2GPI and either protein C or APC targets has not been demonstrated so far. It has also been suggested that β2GPI inhibits factor XII activation and that the inhibition is enhanced by anti-β2GPI [44]. Furthermore, suppression of intrinsic fibrinolytic activity of the euglobulin fraction from normal plasma by β2GPI, independent of factor XII, has been recently described and the effect is reversed by monoclonal anti-β2GPI antibodies in the presence of β2GPI [45]. Thus, β2GPI appears to not only interact with factors of the coagulation cascade, but also participate in the regulation of intrinsic fibrinolysis.

The relationship between β2GPI and factor XII is challenged by the frequent existence of antibodies to FXII in patients with APS [46]. Similarly, antibodies have also been detected against prothrombin, protein C, protein S, and annexin V in patients with SLE-associated APS, moreover as independent, significant risk factors for arterial thrombosis, venous thrombosis, and fetal loss [47]. On the other hand, associations of β2GPI with coagulation factors do not necessarily entail any direct involvement of aPL in the activation of the coagulation cascade for APS-related thrombosis. As long as the exact function of β2GPI remains obscure, the extent of involvement of this molecule in the coagulation cascade, together with the possible implications of the existence of anti-β2GPI antibodies, is difficult to accurately assess (Fig. 1).

β2GPI and pregnancy loss

Recurrent miscarriage constitutes one of the main features of APS [1,2]; however, the pathogenic mechanisms remain unknown and indeed evidence of a causal link has
proven difficult to generate. Early experiments incubating aPL with human placental trophoblast cells showed inhibition of proliferation and secretory activity was elicited by antibodies reacting with β2GPI, confirming the involvement of β2GPI in APS-related pregnancy loss, and suggesting that β2GPI contains the critical antigenic determinants for anticardiolipin antibodies [48]. Alternative hypotheses have also been formulated: (1) aPL may prevent in vitro implantation, after binding to trophectoderm of preimplantation embryos [49]; (2) aPL may interfere with the ability of β2GPI to mediate the activity of lipoprotein lipase [50], for placental prostaglandin synthesis from maternal membrane phospholipids [51]; and (3) β2GPI could react with oxidized-LDL, facilitating the binding of aPL [52]; this induces the uptake of oxidized-LDL by macrophages triggering atherogenesis [53], and acute atherosis is a common finding in the placental bed of complicated pregnancies [54].

Perhaps one of the most attractive potential mechanisms to emerge is the recently described activation of endothelial cells mediated through the annexin II receptor. Binding of aPL to β2GPI on the endothelial cell surface activates genes that result in adhesion molecule, proinflammatory cytokine, and tissue factor up-regulation, ultimately inducing a proinflammatory and a procoagulant phenotype in endothelial cells [55] (Fig. 1). Interestingly, activation mimics the cascade of events elicited by lipopolysaccharide (LPS) or interleukin-1, and transient transfection studies in immortalized endothelial cell lines now implicate a toll-like receptor or interleukin-1 receptor in mediating the NF kappa B-mediated signaling cascade triggered by β2GPI ligation [56].

Experimental data from mouse models on the involvement of aPL in APS-related pregnancy loss are conflicting. Although initially it was postulated that passive transfer of IgG fractions of human anticardiolipin antibodies to pregnant mice results in fetal death [57], such adverse effects of human aPL on murine pregnancy were less successfully reproduced in subsequent, similar studies [58,59]. It could be hypothesized that coadministration of β2GPI to mice (as a contaminant of the IgG preparations) might contribute to this inconsistency. However, conflicting results have also been obtained when mice were immunized with human β2GPI. Immunization with β2GPI did induce aPL in mice; however, it did not result in fetal loss and was not associated with thrombocytopenia [60]. On the contrary, adverse reproductive outcome was associated with β2GPI immunization experiments in a later study, using the same methodology [61]. Recently, complement-mediated placental injury was implicated in passive immunization studies that demonstrated a requirement for complement C3 in fetal loss and growth retardation elicited after injection of human IgG class aPL to pregnant mice [62].

Absence of thrombocytopenia despite fetal loss [52] could support the argument that platelet consumption in placental thrombotic events is not the predominant mechanism of recurrent miscarriages in APS. Even when laboratory data on human APS are reviewed, it is evident that the sole existence of aPL is not sufficient for explaining recurrent fetal loss in human APS: aPL are present in only about one tenth of recurrent aborters [63], while the IgM aCL assay may produce positive but clinically nonsignificant results in high proportions of normal pregnancies [64,65].

The experimental data in mice thus do not clarify whether aPL are associated with pregnancy loss. Key questions remain concerning the specificity of passively transferred human aPL in mice. Some antibody preparations may react only with human β2GPI, while others are also able to recognize epitopes on murine β2GPI or even other molecules. Thus, neither human clinical studies nor animal

Fig. 1. Mechanism of action of anti-β2GPI autoantibodies from APS patients.
models have provided definite evidence for a role of \( \beta_2 \)GPI in pregnancy outcome, and importantly, the extent to which antibodies act through neutralizing function of \( \beta_2 \)GPI versus triggering a \( \beta_2 \)GPI-mediated activation event has not been established.

\( \beta_2 \)GPI—lessons from knockout mice

Mouse models for the role of \( \beta_2 \)GPI in APS

The mouse offers a good model for studying APS. Mice spontaneously develop clinical features of SLE [66] and also manifestations of APS [67,68]. These mice, in addition to SLE type autoantibodies (ANA, anti-ssDNA, and anti-dsDNA) [69,70], develop aCL antibodies that are \( \beta_2 \)GPI dependent [67] and direct anti-B2GPI antibodies [68]. Although initial attempts for inducing SLE in mice were incongruous [71–73], several murine models of APS have now been described. Several groups have replicated aspects of pregnancy pathology in mouse models, albeit with the caveats described above. Moreover, transferred aPL have been reported to confer thrombotic status in mice [74]; however, this effect is technically difficult to estimate accurately, as an important limitation arises from the fact that mice are difficult to venesect repetitively. Treatment of mice, suffering from experimentally induced APS, with IVIG inhibits the thrombogenic effects of aPL in vivo and reduces the levels of aCl in the circulation. Blockade of stimulatory Fc gammaR on inflammatory cells is not necessary for this effect [75].

It must be emphasized that considerable homology in structure exists between human and mouse \( \beta_2 \)GPI [1,76]. This assigns the mouse as a very useful tool for elucidating the role of \( \beta_2 \)GPI, both in homeostasis and in APS. The assumption that a physiological role exists is reinforced by the high degree of conservation of \( \beta_2 \)GPI among mammals [1] together with the abundance of \( \beta_2 \)GPI in plasma [25]. Ontogenetically, either of these two would be unlikely if an important role for \( \beta_2 \)GPI was not allocated. However, as already mentioned, the precise role of \( \beta_2 \)GPI is not fully elucidated: Apart from the “classical” hypothesis on \( \beta_2 \)GPI binding to phospholipids (Fig. 1) interactions of \( \beta_2 \)GPI have been described with coagulation molecules [22,24], annexin II, and other endothelial proteins [77] and also with molecules important for atheroma formation [78]. It is therefore essential that research on \( \beta_2 \)GPI in mice should accommodate the diverse interactions of the protein.

Mice immunized with human \( \beta_2 \)GPI were reported to develop high affinity polyclonal antibodies in both aCL ELISA and then anti-\( \beta_2 \)GPI ELISA [79], probably as a result of nonspecific charge-dependent binding [29]. Immunization of mice with human \( \beta_2 \)GPI results in the generation of antibodies reacting with human, bovine, and murine \( \beta_2 \)GPI. The loss of tolerance to mouse \( \beta_2 \)GPI is attributable to the high interspecies homology of \( \beta_2 \)GPI. Consequently, it was speculated that a molecular mimicry mechanism could be involved in APS [80], postulating that infection may act to trigger initiation of autoimmune process [81,82]. This theory, however, does not entirely explain the common transient occurrence of aPL after infections and the different clinical behavior of this phenomenon, as compared to the APS. Ultimately, further experimental elaboration is required to solidly support such a role for \( \beta_2 \)GPI in the pathogenesis of APS.

Generation of \( \beta_2 \)GPI null mutant mice

New perspectives into research on \( \beta_2 \)GPI have emerged after the recent generation and initial characterization of transgenic mice unable to express \( \beta_2 \)GPI [83], obtained using a homologous recombination approach [76]. Mice with a deletion of the \( \beta_2 \)GPI gene proceed normally to adulthood and show no clear tendency to thrombosis; however, they exhibit impaired thrombin generation upon activation of the extrinsic clotting cascade in defibrinated plasma [83]. The findings from experiments with \( \beta_2 \)GPI-deficient mice on the role of \( \beta_2 \)GPI will be discussed next.

Pregnancy in \( \beta_2 \)GPI null mutant mice

In initial experiments, normal reproductive function was observed in crosses between \( \beta_2 \)GPI-deficient male and female mice [83] with reproductive outcomes indistinguishable from control mice in both males and females carrying the \( \beta_2 \)GPI null mutation in terms of the proportion of animals that bred successfully and the number and viability of pups born at term and surviving to weaning. Furthermore, there was no evidence of altered blood platelet counts in either virgin or pregnant mice. New data from mating of \( \beta_2 \)GPI-deficient mice confirm that \( \beta_2 \)GPI-deficient mice do show any evidence of fetal loss late in gestation and have normal growth trajectories both in utero and after birth (Robertson et al., unpublished observations). Together, these data preclude \( \beta_2 \)GPI from being essential for normal reproductive function in mice.

However, there are observations that suggest homozygote null mutant embryos may be less viable under some circumstances than heterozygote or wild-type littermates. When \( \beta_2 \)GPI heterozygotes on a 129/Sv/C57BL/6 mixed genetic background were intercrossed, only 8.9% of the resulting offspring were homozygous for the deletion, which is significantly lower than the expected 1:2:1 Mendelian ratio [83]. This finding implies that lack of \( \beta_2 \)GPI action confers a selective disadvantage to survival. This is probably the result of a defect at implantation or at even earlier reproductive stages, as litter sizes were comparable in heterozygote and wild-type pregnancies. Furthermore, studies with larger cohorts of null mutant mice have revealed that \( \beta_2 \)GPI deficiency is associated with a 15% reduction in litter size, compared to heterozygote and wild-type pregnancies, along with subtle changes in placental size and structure indicative of impaired placental efficiency (Robertson et al., unpublished observations). Taken together, the above data indicate that \( \beta_2 \)GPI seems to play a facilitating role in the initial stages of pregnancy in mice, but it is still...
unclear if this reflects an impact at the time of ovulation, early embryogenesis, or implantation.

It is difficult to reconcile the observations of essentially normal pregnancy outcomes in β2GPI-deficient mice with negative selection pressure for β2GPI null offspring in heterozygote mating. An explanation for this might be found in the genetic background of the mice used in these experiments, which were hybrids between the C57BL/6 and 129/Sv strains. A reasonable postulate is that at least in part the reproductive phenotype is attributable to genetic modifiers; for example, that the penetrance of the phenotype is a consequence of variance in another gene or genes. While sophisticated strategies would be required to identify such genes, some support for this hypothesis comes from observations that during the initial generation of the null mutant line, only one of four founding transgenic pairs produced progeny over a 3-month breeding interval (S.A. Krulis, unpublished observation), and all subsequent experimental mice were derived from that pair. Furthermore, it might be postulated that the β2GPI status of the mother has an influence on the viability of a β2GPI-deficient conceptus.

The β2GPI null model also promises to provide important insights concerning the role of aPL in pregnancy loss. In preliminary experiments, we have shown that passive transfer into pregnant mice of at least one monoclonal antibody against human β2GPI and one polyclonal human IgG antibody (derived from a patient with APS and associated recurrent miscarriages) causes complete pregnancy loss in a significant proportion of pregnancies. Importantly however, this effect could not be reproduced in pregnant β2GPI-deficient mice, and only some antibodies reactive with β2GPI were effective (Robertson et al., unpublished observations). Perhaps surprisingly, the results show that loss is not associated with placental thrombosis or clotting changes, but rather occurs before or early after embryo implantation. This is of interest in view of the observations of early loss of β2GPI-deficient embryos [83]. These data, combined with the finding that β2GPI is not essential for successful reproductive function, imply that the mechanism of aPL-associated pregnancy loss does not involve deactivation of β2GPI, but rather is the consequence of an event triggered via interaction of aPL with the β2GPI ligand. The result is thus consistent with emerging theories of β2GPI-mediated endothelial cell activation [55] or complement-mediated endothelial cell or trophoblast cell damage [48,62]. The caveat to this interpretation lies in our observation of variable susceptibility of individual mice to treatment with different β2GPI-reactive aPL. It is unclear whether the variance reflects differences among mice in their outbred genetic background, a factor known to be import in thrombotic events [84]. Alternatively, since early pregnancy is notoriously sensitive to environmental stresses such as LPS [85], a further possibility is that exogenous influences interfere with the effect of aPL. Such a scenario seems reasonable in view of the discovery that antibodies binding to β2GPI can activate toll-like receptor-driven NF kappa B signaling [56]. Further studies are therefore required to either validate or reject the hypothesis of β2GPI involvement in early reproductive stages as an underlying pathogenetic mechanism for recurrent fetal loss in APS. Experiments in β2GPI knockout mice, including backcrossing onto a pure C57BL/6 background and investigation of the interaction among β2GPI, aPL, and proinflammatory mediators such as LPS, will help clarify the exact role of β2GPI in reproductive events and APL-mediated fetal loss.

**The coagulation cascade in β2GPI null mutant mice**

While interactions between β2GPI and several factors of the coagulation cascade have been reported, controversy exists about the effect of β2GPI on coagulation. Some important data recently came from studies on β2GPI-deficient mice; these mice show a significantly diminished rate of thrombin generation compared with β2GPI-replete (β2GPI +/+ ) or even with heterozygous mice [83]. Furthermore, the effect of β2GPI on thrombin generation was dose dependent. On the contrary, however, no significant differences in clotting time were observed in plasma from these three genotypes when measured by dRVVT, dKCT, aPTT, and protein C pathway assays. The fact that conventional coagulation assays failed to detect any abnormalities in β2GPI-deficient mice is not inconsistent with the above findings, as these tests measure the time to generate a thrombin-dependent clot, which is a poor indicator of thrombin generation, occurring before peak thrombin production [86].

This interesting effect of β2GPI on thrombin confirmed previous experiments from the same group where prolongation of thrombin generation was observed following the addition of anti-β2GPI antibodies to normal human plasma, regardless of whether the antibody was of mouse monoclonal APS patient origin [87]. Experiments on β2GPI knockout mice gave direct evidence on the role of the protein in the coagulation cascade, at least in one stage upstream thrombin generation. However, it is still unclear if the overall action of β2GPI is procoagulant or anticoagulant since (1) generation of thrombin is important for both thrombus formation and for the initiation of the protein C anticoagulation pathway, and (2) other in vitro interactions of β2GPI with other coagulation factors have been described. It is expected that further research on the mouse model lacking β2GPI will provide clarification.

**Future perspectives**

It is evident that the β2GPI-deficient mouse offers a valuable tool for elucidating the role of β2GPI in homeostasis and in APS. The model has the considerable benefit of allowing the role of this molecule to be interrogated against the complexity of the in vivo environment. In contrast to use of aPL to neutralize β2GPI, the model eliminates the confounding effects of marked heterogeneity in aPL antigen and epitope specificity [88]. Furthermore, null mutant mice
permit experiments to strategically address questions relating to the mechanisms of action underlying the pathogenesis of APS. The mice have already provided new insights into the function of β2GPI in normal and pathological processes and further questions have now emerged. Among these are the following: (1) Is there an effect of genetic background on the penetrance of the β2GPI-deficient phenotype, and if so what are the relevant interacting genes? (2) What stage of conception or early pregnancy is compromised in the β2GPI-deficient phenotype, and if β2GPI-replete mice are differentially affected by β2GPI-reactive aPL? (4) Are there differences in epitope reactivity or avidity that explain why some aPL targeting β2GPI fail to exert any effect on the outcome of murine pregnancies?

Additional challenging questions arise from experiments evaluating the biological function of β2GPI in the coagulation cascade: (1) Is the main action of β2GPI in the coagulation cascade procoagulant or anticoagulant? (2) Is this putative action mediated only by impairment of thrombin generation, given the numerous proposed interactions of β2GPI with coagulation molecules? Furthermore, is there any role for β2GPI in atherogenesis, and how do mice lacking β2GPI behave under various dietary conditions? Ongoing research in β2GPI-deficient mice is expected to contribute significantly to unraveling the role of β2GPI and of antibodies reactive with β2GPI in the pathogenesis of the various clinical manifestations of APS.

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