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

Spyridon Miyakis
University of New South Wales, smiyakis@uow.edu.au

Sarah A. Robertson
University of Adelaide

S A. Krilis
St. George Hospital, Sydney

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

Abstract

The antiphospholipid syndrome is characterized by the presence in serum of autoantibodies against h2GPI. Although the role of h2GPI in the pathogenesis of antiphospholipid antibody syndrome (APS) is well recognized, its exact physiological functions still remain undisclosed. Several interactions of h2GPI with components of the coagulation cascade have been proposed, resulting in both procoagulant and anticoagulant effects. Additionally, h2GPI 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 b2GPI gene has been feasible. h2GPI 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 h2GPI are fertile, the success of early pregnancy is moderately compromised and functional h2GPI is believed necessary for optimal implantation and placental morphogenesis.

Keywords

Disciplines

Medicine and Health Sciences | Social and Behavioral Sciences

Publication Details

CLINICAL IMMUNOLOGY

ELSEVIER

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

Spiros Miyakis,[a] Sarah A. Robertson,[b] and Steven A. Krilis[a],*

[a] Department of Immunology, Allergy and Infectious Disease, St. George Hospital, University of New South Wales, Kogarah, NSW 2217, Australia
[b] Department of Obstetrics and Gynaecology and Reproductive Medicine Unit, University of Adelaide, Adelaide, SA 5005, Australia

Abstract

The antiphospholipid syndrome is characterized by the presence in serum of autoantibodies against β_2GPI. Although the role of β_2GPI in the pathogenesis of antiphospholipid antibody syndrome (APS) is well recognized, its exact physiological functions still remain undisclosed. Several interactions of β_2GPI with components of the coagulation cascade have been proposed, resulting in both procoagulant and anticoagulant effects. Additionally, β_2GPI 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 β_2GPI gene has been feasible. β_2GPI 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 β_2GPI are fertile, the success of early pregnancy is moderately compromised and functional β_2GPI is believed necessary for optimal implantation and placental morphogenesis.
© 2004 Published by Elsevier Inc.

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 β_2 glycoprotein-I (β_2GPI).

The plasma protein β_2GPI is a phospholipid binding protein with anticoagulant properties. β_2GPI 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 β_2GPI 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 β_2GPI-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-β_2GPI antibodies in the sera of APS patients [10,11]. Taken together, these data argue for a key role of β_2GPI in the molecular pathophysiology of APS (summarized in Fig. 1).

β_2 glycoprotein-I

Protein chemistry

β_2GPI 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 β_2GPI 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-β_2GPI autoantibodies [18,19].

The fifth domain of β_2GPI 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 β_2GPI: Site-directed mutagenesis of Lys residues in the lysine-rich region (amino acids 281–288) abolishes the binding of β_2GPI to CL, inhibiting also the binding of antibodies to β_2GPI 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 β_2GPI [20] (Fig. 1). Not surprisingly, therefore, the cleaved β_2GPI 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 β_2GPI 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 β_2GPI [24]. Moreover, heparin at therapeutic concentrations is able to enhance the plasmin-mediated cleavage of the C-terminal region (Lys 317-Thr 318) of β_2GPI [24]. These data indicate the importance of the fifth domain of β_2GPI for binding to anionic phospholipids and heparin.

β_2GPI 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 β_2GPI plasma levels, arising from nucleotide polymorphisms of the *β_2GPI* 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]. β_2GPI is synthesized mainly in the liver [13] and also in intestinal epithelial cells [30] and placenta cells [31]. β_2GPI 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 β_2GPI in vitro binds to anionic phospholipids, producing an apparent anticoagulant effect by occupying the procoagulant binding sites [32]. Interestingly, individuals without detectable β_2GPI have been identified who appear clinically well [33,34].

β_2GPI and coagulation factors

A number of possible in vitro functions of β_2GPI 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 β_2GPI are incongruous [39,40]. Interestingly, β_2GPI-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 β_2GPI in APS-related thrombosis is rather more qualitative than quantitative, with aPL possibly interfering with the physiological functions of β_2GPI, including the protein's interactions with coagulation factors. β_2GPI normally inhibits the generation of factor Xa in the presence of activated platelets, and the presence of anticardiolipin 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 β_2GPI, impairing the ability of β_2GPI to bind phospholipids [21,22]. Moreover, heparin binding to the fifth domain of β_2GPI, at concentrations achieved in vivo, greatly enhances the plasmin-mediated cleavage of β_2GPI [24].

Association of antibodies against β_2GPI with protein C activity has been reported by different groups [40,42,43]; however, a direct interaction between β_2GPI and either protein C or APC targets has not been demonstrated so far. It has also been suggested that β_2GPI inhibits factor XII activation and that the inhibition is enhanced by anti-β_2GPI [44]. Furthermore, suppression of intrinsic fibrinolytic activity of the euglobulin fraction from normal plasma by β_2GPI, independent of factor XII, has been recently described and the effect is reversed by monoclonal anti-β_2GPI antibodies in the presence of β_2GPI [45]. Thus, β_2GPI appears to not only interact with factors of the coagulation cascade, but also participate in the regulation of intrinsic fibrinolysis.

The relationship between β_2GPI 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 β_2GPI 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 β_2GPI remains obscure, the extent of involvement of this molecule in the coagulation cascade, together with the possible implications of the existence of anti-β_2GPI antibodies, is difficult to accurately assess (Fig. 1).

β_2GPI 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
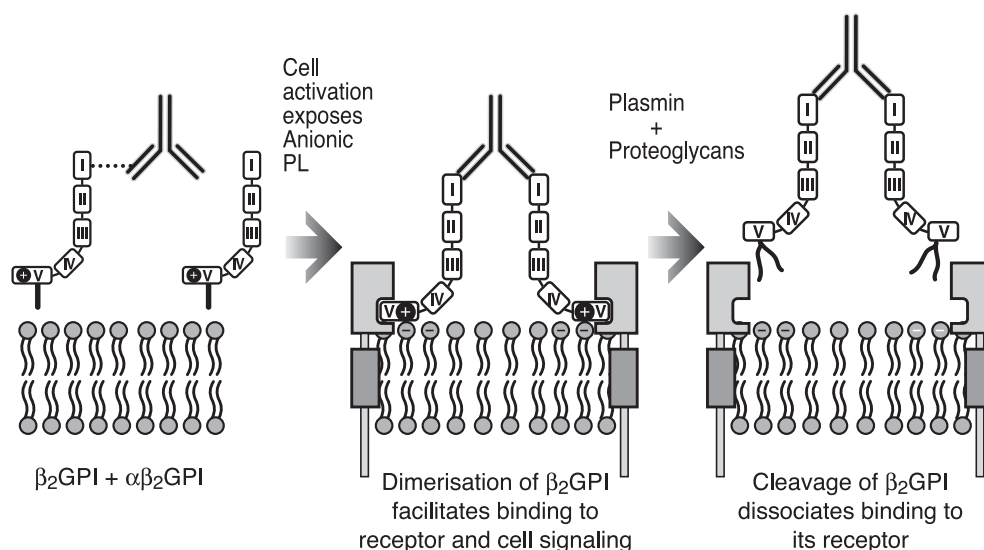
Fig. 1. Mechanism of action of anti-β_2GPI autoantibodies from APS patients.

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 β_2GPI, confirming the involvement of β_2GPI in APS-related pregnancy loss, and suggesting that β_2GPI 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 β_2GPI to mediate the activity of lipoprotein lipase [50], for placental prostaglandin synthesis from maternal membrane phospholipids [51]; and (3) β_2GPI 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 β_2GPI 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 β_2GPI 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 preg-

nant 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 β_2GPI 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 β_2GPI. Immunization with β_2GPI 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 β_2GPI 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 β_2GPI, while others are also able to recognize epitopes on murine β_2GPI or even other molecules. Thus, neither human clinical studies nor animal

models have provided definite evidence for a role of β_2GPI in pregnancy outcome, and importantly, the extent to which antibodies act through neutralizing function of β_2GPI versus triggering a β_2GPI-mediated activation event has not been established.

β_2GPI—lessons from knockout mice

Mouse models for the role of β_2GPI 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 β_2GPI 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 β_2GPI [1,76]. This assigns the mouse as a very useful tool for elucidating the role of β_2GPI, both in homeostasis and in APS. The assumption that a physiological role exists is reinforced by the high degree of conservation of β_2GPI among mammals [1] together with the abundance of β_2GPI in plasma [25]. Ontogenetically, either of these two would be unlikely if an important role for β_2GPI was not allocated. However, as already mentioned, the precise role of β_2GPI is not fully elucidated: Apart from the "classical" hypothesis on β_2GPI binding to phospholipids (Fig. 1) interactions of β_2GPI 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 β_2GPI in mice should accommodate the diverse interactions of the protein.

Mice immunized with human β_2GPI were reported to develop high affinity polyclonal antibodies in both aCL ELISA and then anti-β_2GPI ELISA [79], probably as a result of nonspecific charge-dependent binding [29]. Immunization of mice with human β_2GPI results in the generation of antibodies reacting with human, bovine, and murine β_2GPI. The loss of tolerance to mouse β_2GPI is attributable to the high interspecies homology of β_2GPI. 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 β_2GPI in the pathogenesis of APS.

Generation of β_2GPI null mutant mice

New perspectives into research on β_2GPI have emerged after the recent generation and initial characterization of transgenic mice unable to express β_2GPI [83], obtained using a homologous recombination approach [76]. Mice with a deletion of the β_2GPI 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 β_2GPI-deficient mice on the role of β_2GPI will be discussed next.

Pregnancy in β_2GPI null mutant mice

In initial experiments, normal reproductive function was observed in crosses between β_2GPI-deficient male and female mice [83] with reproductive outcomes indistinguishable from control mice in both males and females carrying the β_2GPI 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 β_2GPI-deficient mice confirm that β_2GPI-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 β_2GPI 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 β_2GPI 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 β_2GPI 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 β_2GPI 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 β_2GPI 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 β_2GPI-deficient mice with negative selection pressure for β_2GPI 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. Krilis, unpublished observation), and all subsequent experimental mice were derived from that pair. Furthermore, it might be postulated that the β_2GPI status of the mother has an influence on the viability of a β_2GPI-deficient conceptus.

The β_2GPI 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 β_2GPI 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 β_2GPI-deficient mice, and only some antibodies reactive with β_2GPI 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 β_2GPI-deficient embryos [83]. These data, combined with the finding that β_2GPI is not essential for successful reproductive function, imply that the mechanism of aPL-associated pregnancy loss does not involve deactivation of β_2GPI, but rather is the consequence of an event triggered via interaction of aPL with the β_2GPI ligand. The result is thus consistent with emerging theories of β_2GPI-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 β_2GPI-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 stressors 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 β_2GPI can activate toll-like receptor-driven NF

kappa B signaling [56]. Further studies are therefore required to either validate or reject the hypothesis of β_2GPI involvement in early reproductive stages as an underlying pathogenetic mechanism for recurrent fetal loss in APS. Experiments in β_2GPI knockout mice, including backcrossing onto a pure C57BL/6 background and investigation of the interaction among β_2GPI, aPL, and proinflammatory mediators such as LPS, will help clarify the exact role of β_2GPI in reproductive events and APL-mediated fetal loss.

The coagulation cascade in β_2GPI null mutant mice

While interactions between β_2GPI and several factors of the coagulation cascade have been reported, controversy exists about the effect of β_2GPI on coagulation. Some important data recently came from studies on β_2GPI-deficient mice; these mice show a significantly diminished rate of thrombin generation compared with β_2GPI-replete (β_2GPI +/+) or even with heterozygous mice [83]. Furthermore, the effect of β_2GPI 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 β_2GPI-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 β_2GPI on thrombin confirmed previous experiments from the same group where prolongation of thrombin generation was observed following the addition of anti-β_2GPI antibodies to normal human plasma, regardless of whether the antibody was of mouse monoclonalor APS patient origin [87]. Experiments on β_2GPI 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 β_2GPI 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 β_2GPI with other coagulation factors have been described. It is expected that further research on the mouse model lacking β_2GPI will provide clarification.

Future perspectives

It is evident that the β_2GPI-deficient mouse offers a valuable tool for elucidating the role of β_2GPI 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 β_2GPI, 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 β_2GPI 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 β_2GPI-deficient phenotype, and if so what are the relevant interacting genes? (2) What stage of conception or early pregnancy is compromised in the absence of β_2GPI? (3) Are there genetic or environmental factors that explain why β_2GPI-replete mice are differentially affected by β_2GPI-reactive aPL? (4) Are there differences in epitope reactivity or avidity that explain why some aPL targeting β_2GPI fail to exert any effect on the outcome of murine pregnancies?

Additional challenging questions arise from experiments evaluating the biological function of β_2GPI in the coagulation cascade: (1) Is the main action of β_2GPI 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 β_2GPI with coagulation molecules? Furthermore, is there any role for β_2GPI in atherogenesis, and how do mice lacking β_2GPI behave under various dietary conditions? Ongoing research in β_2GPI-deficient mice is expected to contribute significantly to unraveling the role of β_2GPI and of antibodies reactive with β_2GPI in the pathogenesis of the various clinical manifestations of APS.

References

[1] D.A. Kandiah, A. Sali, Y. Sheng, E.J. Victoria, D.M. Marquis, S.M. Coutts, S.A. Krilis, Current insights into the "antiphospholipid" syndrome: clinical, immunological, and molecular aspects, Adv. Immunol. 70 (1998) 507–563.

[2] D. Alarcon-Segovia, M.E. Perez-Vazquez, A.R. Villa, C. Drenkard, J. Cabiedes, Preliminary classification criteria for the antiphospholipid syndrome within systemic lupus erythematosus, Semin. Arthritis Rheum. 21 (1992) 275–286.

[3] R.A. Asherson, M.A. Khamashta, J. Ordi-Ros, R.H.M.W. Derksen, S.J. Machin, J. Barquinero, H.H. Outt, E.N. Harris, M. Vilardell-Torres, G.R.V. Hughes, The "primary" antiphospholipid syndrome: major clinical and serological features, Medicine 68 (1989) 366–374.

[4] F. Langer, B. Eifrig, G. Marx, A. Stork, S. Hegewisch-Becker, D.K. Hossfeld, Exacerbation of antiphospholipid antibody syndrome after treatment of localized cancer: a report of two cases, Ann. Hematol. 81 (2002) 727–731.

[5] M. Santiago, R. Martinelli, A. Ko, E.A. Reis, R.D. Fontes, E.G. Nascimento, S. Pierangeli, R. Espinola, A. Gharavi, Anti-beta2 glycoprotein I and anticardiolipin antibodies in leptospirosis, syphilis and kala-azar, Clin. Exp. Rheumatol. 19 (2001) 425–430.

[6] H.P. McNeil, R.J. Simpson, C.N. Chesterman, S.A. Krilis, Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: beta 2-glycoprotein I (apolipoprotein H), Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 4120–4124.

[7] M. Helbert, S. Bodger, J. Cavenagh, D. D'Cruz, J.M. Thomas, P. MacCallum, Optimising testing for phospholipid antibodies, J. Clin. Pathol. 54 (2001) 693–698.

[8] M.A. Audrain, D. El-Kouri, M.A. Hamidou, L. Mioche, A. Ibara, M.L. Langlois, J.Y. Muller, Value of autoantibodies to beta(2)-glycoprotein 1 in the diagnosis of antiphospholipid syndrome, Rheumatology 41 (2002) 550–553.

[9] J.E. Hunt, H.P. McNeil, G.J. Morgan, R.M. Crameri, S.A. Krilis, A phospholipid-beta 2-glycoprotein I complex is an antigen for anticardiolipin antibodies occurring in autoimmune disease but not with infection, Lupus 1 (1992) 75–81.

[10] M. Galli, P. Comfurius, T. Barbui, R.F. Zwaal, E.M. Bevers, Anticoagulant activity of beta 2-glycoprotein I is potentiated by a distinct subgroup of anticardiolipin antibodies, Thromb. Haemost. 68 (1992) 297–300.

[11] R.A. Roubey, C.W. Pratt, J.P. Buyon, J.B. Winfield, Lupus anticoagulant activity of autoimmune antiphospholipid antibodies is dependent upon beta 2-glycoprotein I, J. Clin. Invest. 90 (1992) 1100–1104.

[12] J. Lozier, N. Takahashi, F.W. Putnam, Complete amino acid sequence of human plasma β_2-glycoprotein I, Proc. Natl. Acad. Sci. U.S.A. 81 (1984) 3640–3644.

[13] A. Steinkasserer, C. Estaller, E.H. Weiss, R.B. Sim, A.J. Day, Complete nucleotide and deduced amino acid sequence of human β_2-glycoprotein I, Biochem. J. 277 (1991) 387–391.

[14] L. Patthy, Detecting homology of distantly related proteins with consensus sequences, J. Mol. Biol. 198 (1987) 567–577.

[15] H. Kiato, K. Enjyoji, Amino acid sequence and location of the bisulfide bonds in bovine beta 2 glycoprotein I: the presence of five Sushi domains, Biochemistry 30 (1991) 11687–11694.

[16] R. Schwarzenbacher, K. Zeth, K. Diederichs, A. Gries, G.M. Kostner, P. Laggner, R. Prassl, Crystal structure of human β_2-glycoprotein I: implications for phospholipid binding and the antiphospholipid syndrome, EMBO J. 18 (1999) 6228–6239.

[17] B. Bouma, P.G. de Groot, J.M. van den Elsen, R.B. Ravelli, A. Schouten, M.J. Simmelink, R.H.M.W. Derksen, J. Kroon, P. Gros, Adhesion mechanism of human β_2-glycoprotein I to phospholipids based on its crystal structure, EMBO J. 18 (1999) 5166–5174.

[18] G.M. Iverson, E.J. Victoria, D.M. Marquis, Anti-β_2-glycoprotein I (β_2GPI) autoantibodies recognize an epitope on the first domain of β_2GPI, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 15542–15546.

[19] J. George, B. Gilburd, M. Hojnik, Y. Levy, P. Langevitz, E. Matsuura, T. Koike, Y. Shoenfeld, Target recognition of β_2-glycoprotein I (β_2GPI)-dependent anticardiolipin antibodies: evidence for involvement of the fourth domain of β_2GPI in antibody binding, J. Immunol. 160 (1998) 3917–3923.

[20] Y. Sheng, A. Sali, H. Herzog, J. Lahnstein, S.A. Krilis, Site-directed mutagenesis of recombinant human β_2-glycoprotein I identifies a cluster of lysine residues that are critical for phospholipid binding and anti-cardiolipin antibody activity, J. Immunol. 157 (1996) 3744–3751.

[21] J.E. Hunt, R.J. Simpson, S.A. Krilis, Identification of a region of β_2-glycoprotein I critical for lipid binding and anti-cardiolipin antibody cofactor activity, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 2141–2145.

[22] N. Ohkura, Y. Hagihara, T. Yoshimura, Y. Goto, H. Kato, Plasmin can reduce the function of human beta2 glycoprotein I by cleaving domain V into a nicked form, Blood 91 (1998) 4173–4179.

[23] D.K. Sanghera, D.R. Wagenknecht, J.A. McIntyre, M.I. Kamboh, Identification of structural mutations in the fifth domain of apolipoprotein H (beta 2-glycoprotein I) which affect phospholipid binding, Hum. Mol. Genet. 6 (1997) 311–316.

[24] J. Guerin, Y. Sheng, S. Reddel, G.M. Iverson, M.G. Chapman, S.A. Krilis, Heparin inhibits the binding of beta 2-glycoprotein I to phospholipids and promotes the plasmin-mediated inactivation of this blood protein. Elucidation of the consequences of the two biological events in patients with the anti-phospholipid syndrome, J. Biol. Chem. 277 (2002) 2644–2649.

[25] T. McNally, I.J. Mackie, D.A. Isenberg, S.J. Machin, Immunoelectrophoresis and ELISA techniques for assay of plasma β_2-glycoprotein-1 and the influence of plasma lipids, Thromb. Res. 72 (1993) 275–286.

[26] H. Mehdi, C.E. Aston, D.K. Sanghera, R.F. Hamman, M.I. Kamboh, Genetic variation in the apolipoprotein H (beta2-glycoprotein I) gene affects plasma apolipoprotein H concentrations, Hum. Genet. 105 (1999) 63–71.

[27] H. Mehdi, S. Manzi, P. Desai, Q. Chen, C. Nestlerode, F. Bontempo, S.C. Strom, R. Zarnegar, M.I. Kamboh, A functional polymorphism at the transcriptional initiation site in beta2-glycoprotein I (apolipoprotein H) associated with reduced gene expression and lower plasma levels of beta2-glycoprotein I, Eur. J. Biochem. 270 (2003) 230–238.

[28] N.S. Lee, H.B.J. Brewer, J.C. Osborne Jr., β_2-Glycoprotein I. Molecular properties of an unusual apolipoprotein, apolipoprotein H, J. Biol. Chem. 258 (1983) 4765–4770.

[29] Y. Nakaya, E.J. Schaefer, H.B. Brewer Jr., Activation of human post heparin lipoprotein lipase by apolipoprotein H (β_2-glycoprotein I), Biochem. Biophys. Res. Commun. 95 (1980) 1168–1172.

[30] M. Averna, G. Paravizzini, G. Marino, E. Lanteri, G. Cavera, C.M. Barbagallo, S. Petralia, S. Cavallaro, G. Magro, S. Grasso, A. Notarbartolo, S. Travali, Liver is not the unique site of synthesis of beta 2-glycoprotein I (apolipoprotein H): evidence for an intestinal localization, Int. J. Clin. Lab. Res. 27 (1997) 207–212.

[31] L.W. Chamley, J.L. Allen, P.M. Johnson, Synthesis of beta2 glycoprotein 1 by the human placenta, Placenta 18 (1997) 403–410.

[32] S.W. Reddel, S.A. Krilis, The anti-phospholipid syndrome, in: A.N. Theofilopoulos, C.A. Bona (Eds.), The Molecular Pathology of Autoimmune Diseases, second ed., Taylor and Francis, New York, 2002, pp. 325–352.

[33] J.M. Hoeg, P. Segal, R.E. Gregg, Y.S. Chang, F.T. Lindgren, G.L. Adamson, M. Frank, C. Brickman, H.B. Brewer Jr., Characterization of plasma lipids and lipoproteins in patients with beta 2-glycoprotein I (apolipoprotein H) deficiency, Atherosclerosis 55 (1985) 25–34.

[34] S. Yasuda, A. Tsutsumi, H. Chiba, H. Yanai, Y. Miyoshi, R. Takeuchi, T. Horita, T. Atsumi, K. Ichikawa, E. Matsuura, T. Koike, Beta(2)-glycoprotein I deficiency: prevalence, genetic background and effects on plasma lipoprotein metabolism and hemostasis, Atherosclerosis 152 (2000) 337–346.

[35] J. Nimpf, E.M. Bevers, P.H. Bomans, U. Till, H. Wurm, G.M. Kostner, R.F. Zwaal, Prothrombinase activity of human platelets is inhibited by beta 2-glycoprotein-I, Biochim. Biophys. Acta 884 (1986) 142–149.

[36] T. Mori, H. Takeya, J. Nishioka, E.C. Gabazza, K. Suzuki, β_2-Glycoprotein I modulates the anticoagulant activity of activated protein C on the phospholipid surface, Thromb. Haemost. 75 (1996) 49–55.

[37] J. Nimpf, H. Wurm, M. Kostner, Interaction of β_2-glycoprotein I with human blood platelets: influences upon the ADP induced aggregation, Thromb. Haemost. 54 (1985) 397–401.

[38] W. Shi, B.H. Chong, P.J. Hogg, C.N. Chesterman, Anticardiolipin antibodies block the inhibition by β_2-glycoprotein I of the factor Xa generating activity of platelets, Thromb. Haemost. 70 (1993) 342–345.

[39] O. Amengual, T. Atsumi, M.A. Khamashta, G.R.V. Hughes, The role of the tissue factor pathway in the hypercoagulable state in patients with the antiphospholipid syndrome, Thromb. Haemost. 79 (1998) 276–281.

[40] M. Ieko, K. Ichikawa, D.A. Triplett, E. Matsuura, T. Atsumi, K. Sawada, T. Koike, Beta2-glycoprotein I is necessary to inhibit protein C activity by monoclonal anticardiolipin antibodies, Arthritis Rheum. 42 (1999) 167–174.

[41] R. Takeuchi, T. Atsumi, M. Ieko, H. Takeya, S. Yasuda, K. Ichikawa, A. Tsutsumi, K. Suzuki, T. Koike, Coagulation and fibrinolytic activities in 2 siblings with beta2-glycoprotein I deficiency, Blood 96 (2000) 1594–1595.

[42] J.D. Oosting, R.H. Derksen, I.W. Bobbink, T.M. Hackeng, B.N. Bouma, P.G. de Groot, Antiphospholipid antibodies directed against a combination of phospholipids with prothrombin, protein C, or protein S: an explanation for their pathogenic mechanism? Blood 81 (1993) 2618–2625.

[43] M. Galli, L. Ruggeri, T. Barbui, Differential effects of anti-beta2-glycoprotein I and antiprothrombin antibodies on the anticoagulant activity of activated protein C, Blood 91 (1998) 1999–2004.

[44] I. Schousboe, M.S. Rasmussen, Synchronized inhibition of the phospholipid mediated autoactivation of factor XII in plasma by b2-glycoprotein I and anti-b2-glycoprotein I, Thromb. Haemost. 73 (1995) 798–804.

[45] R. Takeuchi, T. Atsumi, M. Ieko, Y. Amasaki, K. Ichikawa, T. Koike, Suppressed intrinsic fibrinolytic activity by monoclonal anti-beta-2 glycoprotein I autoantibodies: possible mechanism for thrombosis in patients with antiphospholipid syndrome, Br. J. Haematol. 119 (2002) 781–788.

[46] D.W. Jones, I.J. MacKie, M.J. Gallimore, M. Winter, Antibodies to factor XII and recurrent fetal loss in patients with the anti-phospholipid syndrome, Br. J. Haematol. 113 (2001) 550–552.

[47] J. Nojima, H. Kuratsune, E. Suehisa, Y. Futsukaichi, H. Yamanishi, T. Machii, Y. Iwatani, Y. Kanakura, Association between the prevalence of antibodies to beta(2)-glycoprotein I, prothrombin, protein C, protein S, and annexin V in patients with systemic lupus erythematosus and thrombotic and thrombocytopenic complications, Clin. Chem. 47 (2001) 1008–1015.

[48] L.W. Chamley, A.M. Duncalf, M.D. Mitchell, P.M. Johnson, Action of anticardiolipin and antibodies to beta2-glycoprotein-I on trophoblast proliferation as a mechanism for fetal death, Lancet 352 (1998) 1037–1038.

[49] Z.M. Sthoeger, E. Mozes, B. Tartakovsky, Anti-cardiolipin antibodies induce pregnancy failure by impairing embryonic implantation, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 6464–6467.

[50] Y. Nakaya, E.J. Schaefer, H.B. Brewer Jr., Activation of human post heparin lipoprotein lipase by apolipoprotein H (β_2-glycoprotein I), Biochem. Biophys. Res. Commun. 95 (1980) 1168–1172.

[51] D.A. Chappel, A.A. Spector, Lipoprotein and lipid metabolism: basis and clinical aspects, in: G.P. Redmond (Ed.), Lipids and Women's Health, Springer-Verlag, New York, 1991, pp. 21–38.

[52] E. Matsuura, Y. Igarashi, T. Yasuda, D.A. Triplett, T. Koike, Anticardiolipin antibodies recognize β_2-glycoprotein I structure altered by interacting with an oxygen modified solid phase surface, J. Exp. Med. 179 (1994) 457–462.

[53] Y. Hasunuma, E. Matsuura, Z. Makita, T. Katahira, S. Nishi, T. Koike, Involvement of β_2-glycoprotein I and anticardiolipin antibodies in oxidatively modified low-density lipoprotein uptake by macrophages, Clin. Exp. Immunol. 107 (1997) 569–573.

[54] C.W. Redman, Pre-eclampsia and the placenta, Placenta 12 (1991) 301–308.

[55] P. Riboldi, M. Gerosa, E. Raschi, C. Testoni, P.L. Meroni, Endothelium as a target for antiphospholipid antibodies, Immunobiology 207 (2003) 29–36.

[56] E. Raschi, C. Testoni, D. Bosisio, M.O. Borghi, T. Koike, A. Mantovani, P.L. Meroni, Role of the MyD88 transduction signaling pathway in endothelial activation by antiphospholipid antibodies, Blood 101 (2003) 3495–3500.

[57] D.W. Branch, D.J. Dudley, M.D. Mitchell, K.A. Creighton, T.M. Abbott, E.H. Hammond, R.A. Daynes, Immunoglobulin G fractions from patients with antiphospholipid antibodies cause fetal death in BALB/c mice: a model for autoimmune fetal loss, Am. J. Obstet. Gynecol. 163 (1990) 210–216.

[58] M. Blank, J. Cohen, V. Toder, Y. Shoenfeld, Induction of anti-phospholipid syndrome in naive mice with mouse lupus monoclonal and human polyclonal anti-cardiolipin antibodies, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 3069–3073.

[59] L.W. Chamley, N.S. Pattison, E.J. McKay, The effect of human anti-cardiolipin antibodies on murine pregnancy, J. Reprod. Immunol. 27 (1994) 123–134.

[60] R.M. Silver, S.S. Pierangeli, A.E. Gharavi, E.N. Harris, S.S. Edwin, C.M. Salafia, D.W. Branch, Induction of high levels of anticardiolipin antibodies in mice by immunization with beta 2-glycoprotein I does not cause fetal death, Am. J. Obstet. Gynecol. 173 (1995) 1410–1415.

[61] C.O. Garcia, A. Kanbour-Shakir, H. Tang, J.F. Molina, L.R. Espinoza, A.E. Gharavi, Induction of experimental antiphospholipid antibody syndrome in PL/J mice following immunization with beta 2 GPI, Am. J. Reprod. Immunol. 37 (1997) 118–124.

[62] V.M. Holers, G. Girardi, L. Mo, J.M. Guthridge, H. Molina, S.S. Pierangeli, R. Espinola, L.E. Xiaowei, D. Mao, C.G. Vialpando, J.E. Salmon, Complement C3 activation is required for antiphospholipid antibody-induced fetal loss, J. Exp. Med. 195 (2002) 211–220.

[63] R.S. Rai, L. Regan, K. Clifford, W. Pickering, M. Dave, I. Mackie, T. McNally, H. Cohen, Antiphospholipid antibodies and β_2-glycoprotein-I in 500 women with recurrent miscarriage: results of a comprehensive screening approach, Hum. Reprod. 10 (1995) 2001–2005.

[64] N.S. Pattison, L.W. Chamley, E.J. McKay, G.C. Liggins, W.S. Butler, Antiphospholipid antibodies in pregnancy: prevalence and clinical associations, Br. J. Obstet. Gynaecol. 100 (1993) 909–913.

[65] P.V. Bagger, V. Andersen, B. Baslund, B. Beck, H. Hove, M. Hoier-Madsen, J. Petersen, J. Philip, O. Schaadt, S.O. Skouby, Anti-cardiolipin antibodies (IgG and IgA) in women with recurrent fetal loss correlate to clinical and serological characteristics of SLE, Acta Obstet. Gynecol. Scand. 72 (1993) 465–469.

[66] A.N. Theofilopoulos, F.J. Dixon, Murine models of systemic lupus erythematosus, Adv. Immunol. 37 (1985) 269–390.

[67] T. Koike, Anticardiolipin antibodies in NZW × BXSB F1 mice, Lupus 3 (1994) 241–246.

[68] M. Monestier, D.A. Kandiah, S. Kouts, K.E. Novick, G.L. Ong, M.Z. Radic, S.A. Krilis, Monoclonal antibodies from NZW × BXSB F1 mice to β_2-glycoprotein I and cardiolipin. Species specificity and charge-dependent binding, J. Immunol. 156 (1996) 2631–2641.

[69] M.G. Cohen, K.M. Pollard, L. Schrieber, Relationship of age and sex to autoantibody expression in MRL-+/+ and MRL-lpr/lpr mice: demonstration of an association between the expression of antibodies to histones, denatured DNA and Sm in MRL-+/+ mice, Clin. Exp. Immunol. 72 (1988) 50–54.

[70] A.E. Gharavi, R.C. Mellors, K.B. Elkon, IgG anti-cardiolipin antibodies in murine lupus, Clin. Exp. Immunol. 78 (1989) 233–238.

[71] S. Mendlovic, S. Brocke, Y. Shoenfeld, M. Ben-Bassat, A. Meshorer, R. Bakimer, E. Mozes, Induction of a systemic lupus erythematosus-like disease in mice by a common human anti-DNA idiotype, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 2260–2264.

[72] M. Blank, J. Cohen, V. Toder, Y. Shoenfeld, Induction of anti-phospholipid syndrome in naive mice with mouse lupus monoclonal and human polyclonal anti-cardiolipin antibodies, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 3069–3073.

[73] D.A. Isenberg, D. Katz, S. Le Page, B. Knight, L. Tucker, P. Maddison, P. Hutchings, R. Watts, J. Andre-Schwartz, R.S. Schwartz, Independent analysis of the 16/6 idiotype lupus model. A role for an environmental factor? J. Immunol. 147 (1991) 4172–4177.

[74] S.S. Pierangeli, E.N. Harris, Antiphospholipid antibodies in an in vivo thrombosis model in mice, Lupus 3 (1994) 247–251.

[75] S.S. Pierangeli, R. Espinola, X. Liu, E.N. Harris, J.E. Salmon, Identification of an Fc gamma receptor-independent mechanism by which intravenous immunoglobulin ameliorates antiphospholipid antibody-induced thrombogenic phenotype, Arthritis Rheum. 44 (2001) 876–883.

[76] Y. Sheng, H. Herzog, S.A. Krilis, Cloning and characterization of the gene encoding the mouse β_2-glycoprotein I, Genomics 41 (1997) 128–130.

[77] K. Ma, R. Simantov, J.C. Zhang, R. Silverstein, K.A. Hajjar, K.R. McCrae, High affinity binding of beta 2-glycoprotein I to human endothelial cells is mediated by annexin II, J. Biol. Chem. 275 (2000) 15541–15548.

[78] S. Kochl, F. Fresser, E. Lobentanz, G. Baier, G. Utermann, Novel interaction of apolipoprotein(a) with β_2-glycoprotein I mediated by the kringle IV domain, Blood 90 (1997) 1482–1489.

[79] A.E. Gharavi, L.R. Sammaritano, J. Wen, K.B. Elkon, Induction of antiphospholipid autoantibodies by immunization with β_2-glycoprotein I (apolipoprotein H), J. Clin. Invest. 90 (1992) 1105–1109.

[80] A. Tincani, B. Gilburd, M. Abu-Shakra, M. Blank, F. Allegri, R. Ottaviani, M. Riboni, P.L. Meroni, G. Balestrieri, Y. Shoenfeld, Immunization of naive BALB/c mice with human beta2-glycoprotein I breaks tolerance to the murine molecule, Arthritis Rheum. 46 (2002) 1399–1404.

[81] E.E. Gharavi, H. Chaimovich, E. Cucurull, C.M. Celli, H. Tang, W.A. Wilson, A.E. Gharavi, Induction of antiphospholipid antibodies by immunization with synthetic viral and bacterial peptides, Lupus 8 (1999) 449–455.

[82] M. Blank, I. Krause, M. Fridkin, N. Keller, J. Kopolovic, I. Goldberg, A. Tobar, Y. Shoenfeld, Bacterial induction of autoantibodies to beta2-glycoprotein-I accounts for the infectious etiology of antiphospholipid syndrome, J. Clin. Invest. 109 (2002) 797–804.

[83] Y. Sheng, S.W. Reddel, H. Herzog, Y.X. Wang, T. Brighton, M.P. France, S.A. Robertson, S.A. Krilis, Impaired thrombin generation in β_2-glycoprotein I null mice, J. Biol. Chem. 276 (2001) 13817–13821.

[84] A. Zumbach, G.A. Marbet, D.A. Tsakiris, Influence of the genetic background on platelet function, microparticle and thrombin generation in the common laboratory mouse, Platelets 12 (2001) 496–502.

[85] J.B. Bishop, R.W. Morris, J.C. Seely, L.A. Hughes, K.T. Cain, W.M. Generoso, Alterations in the reproductive patterns of female mice exposed to xenobiotics, Fundam. Appl. Toxicol. 40 (1997) 191–204.

[86] M.D. Rand, J.B. Lock, C. van't Veer, D.P. Gaffney, K.G. Mann, Blood clotting in minimally altered whole blood, Blood 88 (1996) 3432–3445.

[87] J.G. Hanly, D. Kandiah, S. Kouts, Y. Sheng, T. Koike, K. Ichikawa, M. Monestier, S. Krilis, Detection of "antiphospholipid antibodies" using a chromogenic assay of thrombin generation, Lupus 7 (1998) S219.

[88] P. Lieby, A. Soley, H. Levallois, B. Hugel, J.M. Freyssinet, M. Cerutti, J.L. Pasquali, T. Martin, The clonal analysis of anticardiolipin antibodies in a single patient with primary antiphospholipid syndrome reveals an extreme antibody heterogeneity, Blood 97 (2001) 3820–3828.