THROMBOGENICITY OF BETA-2-GLYCOPROTEIN I
DEPENDENT ANTIPHOSPHOLIPID ANTIBODIES IN
A PHOTOCHEMICALLY INDUCED THROMBOSIS
MODEL IN THE HAMSTER

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Summary

We previously showed that β2GPI-dependent lupus anticoagulants (LA) form bivalent antigen-antibody complexes with high affinity for phospholipids; these complexes are responsible for their in vitro anticoagulant effect. We now studied the role of these bivalent complexes in arterial thrombosis in the hamster. Three monoclonal antibodies (Mab) raised against human β2GPI were selected on the basis of their cross-reactivity with hamster β2GPI. Two of these, one with LA activity (5H2) and one with only anticardiolipin properties (11E8), were infused at 0 to 10 mg/kg prior to photochemically-induced vessel damage. 5H2 promoted thrombus formation dose-dependently, raising the thrombus size from 6.0 arbitrary units (AU) in controls (n=9) to 65.0 AU in the high dose group (10 mg/kg, n=6, p=0.007). The LA negative Mab 11E8 and Mab 27A8, reactive with human β2GPI exclusively, did not significantly promote thrombus formation. In a second set of experiments, intact Mab 5H2 was compared to its fragments. Intact Mab 5H2 at 3.3 mg/kg and the equimolar dose of F(ab’)2 fragments (2.2 mg/kg), promoted thrombus formation equally well (55.8 AU, n=8 and 62.5 AU, n=7 respectively); Mab 5H2 derived Fab’ fragments were inactive. Immunohistochemical analysis showed platelet-rich thrombi, with 5H2 or its F(ab’)2 fragments mainly bound to individual platelets. Our results indicate that bivalent immune complex formation plays an important role in the genesis of arterial thrombosis by certain aPL. Cellular activation via the Fc portion of these immune complexes however is not essential, since F(ab’)2 fragments of 5H2 still promote thrombus formation.
Introduction

Antiphospholipid antibodies (aPL) are a heterogeneous group of immunoglobulins interacting with negatively charged phospholipids. They are found in serum or plasma of patients with rheumatic diseases, malignancies or infections, but also in apparently healthy individuals. Persistently elevated aPL levels are associated with the occurrence of arterial and venous thrombosis, thrombocytopenia and recurrent fetal loss (1). This clinical entity, referred to as the antiphospholipid syndrome (APS), is considered secondary or primary respectively in subjects with or without systemic autoimmune diseases, e.g. systemic lupus erythematosus (SLE) (2). Paradoxically, an important subset of aPL, termed lupus anticoagulants (LA), prolongs in vitro plasma clotting times (3, 4).

The first immunoassays for the detection of aPL made use of cardiolipin (5, 6). However, the so-called anticardiolipin antibodies (aCL) also bind to other negatively charged phospholipids (PL), such as phosphatidylserine (7). Affinity purification of aCL revealed that aCL binding to cardiolipin depends on a plasma protein, β2-glycoprotein I (β2GPI) (8-10). It is now generally accepted that autoimmune aPL are directed against proteins binding to anionic phospholipid surfaces rather than against phospholipids themselves, the major protein targets appearing to be β2GPI and prothrombin (11, 12). LA positive aPL crosslink two β2GPI molecules or two prothrombin molecules and thereby induce the correct spatial orientation of the phospholipid binding domains in these proteins required for optimal binding to PL (13-16). The dimerized β2GPI or prothrombin display a marked gain in affinity for the phospholipid surface and retard clotting in vitro by competing with clotting factors for the same phospholipid surface.

Autoimmune aPL are thought to be pathogenic as patients with aPL not only have an increased risk for thrombosis but also show signs of a prothrombotic (hypercoaguable) state with elevated tissue factor (TF) expression (17) and enhanced thrombin generation (18). The mechanism(s) by which these antibodies cause a prothrombotic state or promote thrombosis is (are) still far from being elucidated. Several hypotheses have been proposed including a decreased prostacyclin formation by endothelium, inhibition of protein C activation or of activated protein C function, impairment of TF inhibition, interference with the function of antithrombin, impaired fibrinolytic potential, reduced anticoagulant potential of annexin V and activation of platelets (reviewed in 19). However, none of these hypotheses explain why
thrombosis can be venous as well as arterial and why LA are more strongly associated with thrombosis than aCL (20, 21).

Analogous to heparin-induced thrombocytopenia, another syndrome of antibody-mediated thrombosis, a model of prothrombotic cellular activation was proposed. Limited damage or activation of blood cells or endothelium may cause exposure of anionic phospholipids on the cell surface. In the presence of aPL with LA activity, bivalent antigen-antibody complexes may form on these cell membranes enriched in anionic phospholipid. These complexes may then bind to cellular Fcγ receptors or activate the complement system leading to strong thrombosis-promoting cell activation via release of granule contents and of microvesicles, thromboxane A2 biosynthesis, tissue factor decryption, removal of endothelial heparan sulphate, etc. (22, 23).

In the present study, this hypothesis was tested in a hamster model of arterial thrombosis (24), adapted in our laboratory to study prothrombotic phenotypes (25). A murine Mab against human β2GpI with clear lupus anticoagulant activity and cross-reacting with hamster β2GpI was selected. The consequences on photochemically induced platelet-rich thrombosis in the hamster were then studied after injection of intact antibody, or its F(ab')2, respectively Fab’ fragments. Our findings show that aPL-associated thrombosis, in contrast to HIT, can occur independently of Fc.

Material and Methods

Monoclonal antibodies against β2GPI

The monoclonal antibodies (Mabs) against human β2GPI, previously raised in Balb/c mice (15), were used in this study. These Mabs were purified from ascites by affinity chromatography on protein A-Sepharose (26).

Preparation of F(ab’)2 fragments

Purified Mab 5H2 at 3 mg/ml was dialyzed overnight at 4°C against 100 mmol/L sodium citrate buffer, pH 3.5. Digestion was performed by addition of 5 µg pepsin beads (Sigma, St. Louis, MO, USA) per mg Mab. After incubation for 60 min at 20°C, the beads were
Preparation of Fab’ fragments

Purified Mab 5H2 at 3 mg/ml was dialyzed overnight at 4°C against 100 mmol/L phosphate buffer, pH 7.0 after which 10 mmol/L cysteine and 2 mmol/L EDTA were added. Digestion was performed by addition of 30 μg papain beads (Sigma) per mg Mab. After incubation at 37°C for 60 min, the reaction was stopped by 75 mmol/L iodoacetamide (Sigma). Following dialysis against 100 mmol/L Tris-HCl buffer pH 8.3, intact Fc fragments and non-digested antibody were removed by protein A Sepharose chromatography. The Fab’ preparation showed a single band at 50 kDa and 25 kDa in unreduced and reduced SDS-PAGE and silver staining respectively.

Reactivity of Mabs with hamster β2GPI

To assess the cross-reactivity of the anti-human β2GPI Mabs with hamster β2GPI, microtiter plates (Costar; Corning Inc., NY, USA) were incubated with 50 μl/well of phosphatidylserine (Sigma) dissolved in absolute ethanol (27 μg/ml) and evaporated overnight at 4°C. The plates were then blocked with 5% hamster plasma in PBS, as a source of β2GPI, for 60 min at room temperature. Anti-β2GPI Mabs dissolved in 1% hamster plasma in PBS (0.5 to 10 μg/ml) were added to the plate and incubated for 120 min at room temperature. Plates were then washed 3 times with PBS and incubated for 120 min with 100 μl/well horse-radish peroxidase coupled goat anti-mouse immunoglobulins (GAM-HRP; Dako, Denmark) diluted 1:3000 in PBS, containing 1% hamster plasma. After washing, 160 μl of 100 mmol/L citrate-200 mmol/L sodium phosphate buffer containing O-Phenylenediamine (OPD; Fluka, Buchs, Switzerland) and 0.003% H2O2 was added to each well. After approximately 30 min at room temperature, staining was stopped with 50 μl of 4 mol/L H2SO4. A (490 nm) was then
measured with a multiscan spectrophotometer (ELx808, Bio-Tek Instruments, Inc., Winooski, VT, USA).

**Lupus anticoagulant activity of anti-β₂GPI Mabs in hamster plasma**

Determination of lupus anticoagulant (LA) activity was based on its effect on the dilute prothrombin time (dPT) (27). Platelet poor plasma (PPP) was prepared by double centrifugation from hamster blood collected on trisodium citrate. The anti-β₂GPI Mabs were 10 fold diluted in this PPP to achieve final concentrations between 0 and 150 µg/ml and incubated for 10 min at 37°C prior to testing. The dPT was determined by incubating 50 µl Innovin (DADE, Switzerland) diluted 1:200 in Owrens Veronal buffer, with 50 µl PPP containing Mab for 7 min at 37°C after which coagulation was initiated by adding 50 µl CaCl₂ (25 mmol/l). Coagulation times were measured using a SYSMEX CA 6000 coagulometer (TOA medical instruments, Japan).

**Affinity for β₂GPI of intact 5H2 and its F(ab’)_2 and Fab’ fragments**

Microtiter plates were coated overnight at 4°C with 50 µl purified human β₂GPI at 5 µg/ml. The plates were blocked with 1% fatty acid free bovine serum albumin fraction V (BSA; Boehringer Mannheim, Germany) and washed 3 times with PBS containing 0.1% Tween 20 (Merck, Hohenbrunn, Germany). Intact 5H2 or its F(ab’)₂ or Fab’ fragments (0 to 3 µg/ml PBS), were applied and incubated for 120 min at 20°C. After washing, antibody-β₂GPI complexes were detected by adding GAM-HRP diluted 1:6000 in 0.1% BSA using OPD as substrate.

**Induction of β₂-GPI binding to phospholipids by intact 5H2 and its F(ab’)₂ or Fab’ fragments.**

Microtiter plates were incubated overnight at 4 °C with 50 µl/well of a mixture containing 75% phosphatidylcholine (Sigma) and 25% phosphatidylserine (Sigma) dissolved in absolute ethanol (total concentration 27 µg/ml). The plates were blocked with 1% β₂GPI-free BSA and washed 3 times with PBS. 5H2 and its F(ab’)₂ or Fab’ (0 to 10 µg/ml), diluted in 0.2% hamster plasma, were added to the phospholipid coated plates for 120 min at 20°C. After
washing, bound hamster $\beta_2$GPI was detected using peroxidase coupled rabbit polyclonal anti-$\beta_2$GPI antiserum (home made) diluted 1:3000 in 0.1% BSA.

Inhibition of intact 5H2-induced hamster $\beta_2$GPI binding to phospholipids by Fab’ fragments

Intact 5H2, added to diluted hamster plasma at a fixed concentration of 625 ng/ml, was preincubated for 45 min at 20 °C with its Fab’ fragments (0 to 20 µg/ml) and added to microtiter plates coated with phosphatidylcholine and phosphatidylerine, as indicated above. After incubation for 120 min at 20 °C, bound hamster $\beta_2$GPI was detected as above.

Photochemically induced thrombosis model in the hamster

All animal experiments were reviewed and approved by the Institutional Review Board of the University of Leuven and were performed in accordance to protocols approved by the Institutional Animal Care and Research Advisory Committee. Male hamsters (Pdf gold, University of Leuven, Belgium) weighing 100-130 g were anesthetized by an intraperitoneal injection of sodium pentobarbital (Nembutal, Sanofi Animal Care, Brussels, Belgium) at a dose of 60 mg/kg and then fixed on a thermostated operation table. A 2.5F venous catheter (Portex, Hythe, UK) was inserted into the right jugular vein. The left carotid artery was carefully dissected from surrounding tissue and mounted on a transilluminator. Thrombus formation was induced by a photochemical reaction according to the method of Umemura et al. (24). Briefly, just after injection of Rose-Bengal (Sigma) at a dose of 20 mg/kg, the exposed artery was irradiated for 2 min with green light (wavelength 540 nm) from a Xenon lamp (L4887, Hamamatsu Photonics, Hamamatsu, Japan) equipped with a heat-absorbing filter and a green filter. Irradiation was directed via a 3-mm diameter optic fiber attached to a manipulator. All tested reagents were administered via an intravenous (slow) bolus injection prior to Rose-Bengal. Intact Mabs and F(ab’)2 fragments were given 15 min before photochemical vessel injury, whereas Fab’ and buffer injections just preceded Rose-Bengal. Quantification of mural thrombi in the hamster carotid artery was performed as described with minor modifications (28). Thrombus formation in the injured transilluminated vessel was constantly monitored for 40 min via a camera (CV-M70, JAI Corporation, Japan) mounted on a microscope. The images were digitized with an image processing software (Optimas 6.5 for Windows 95/98 & NT 4.0, Media Cybernetics, Silver Spring, MD, USA with a specific
extension from I.P. Consult, Breda, the Netherlands) and constantly recorded. The transmitted light intensity versus time curve was established and thrombus formation was measured by comparing the area under the curve, expressed in arbitrary light units (AU).

**Determination of the concentration of intact 5H2 and its F(ab’)2 fragments in hamster plasma**

Plasma was prepared from blood drawn via the intravenous catheter just after completion of the *in vivo* experiments. Plasma concentrations of 5H2 or its F(ab’)2 fragments were measured by ELISA as follows. Microtiter plates were coated overnight at 4°C with 200 µl/well of polyclonal rabbit anti-mouse IgG (5 µg/ml), blocked with 1% BSA and washed 3 times with PBS containing 0.1% Tween 20. Hamster plasma samples, 1:2000 and 1:1000 diluted in PBS for measurement of intact 5H2 and its F(ab’)2 fragments, respectively, were added and incubated for 120 min at 20°C. Standard curves were constructed using 5H2 or 5H2 derived fragment solutions (0 to 200 µg/ml in hamster plasma), diluted in the same way as the ex vivo samples. After washing, bound Mabs were detected by GAM-HRP diluted 1:3000 in PBS, containing hamster and rabbit plasma (1/300) to adsorb goat anti-mouse antibodies cross-reacting with hamster and rabbit antibodies. The HRP activity was determined with OPD as a substrate.

**Immunohistochemistry**

Carotid arteries containing thrombi were carefully dissected, fixed overnight at 4°C in 4% formaldehyde in PBS, pH 7.0, and transferred to PBS containing 20% sucrose for 24 hrs. Arteries were embedded in OTC (Tissue-Tec, Milses Inc), snap-frozen in precooled 2-methyl butane and stored at -70°C until further analysis. Seven-micron-thick sections were made through the whole thrombosed artery for hematoxylin-eosin staining. Immunohistochemical staining for the presence of 5H2 and its F(ab’)2 fragments was done with a GAM-HRP diluted 1:250 in TBS containing 2% BSA and preincubated for 30 min with 10% hamster plasma to adsorb nonspecific antibodies. Peroxidase staining was performed in 50 mmol/L Tris-HCl buffer, pH 7.0, containing 0.06% 3,3-diaminobenzidine and 0.01% H2O2. Tissue sections were counterstained with hematoxylin.
Platelet aggregation studies

Blood for platelet aggregation studies was freshly drawn from healthy donors on 109 mM trisodium citrate and centrifuged at 150 g for 15 min. The platelet-rich plasma (PRP) was collected and the platelet counts were adjusted to $2.5 \times 10^5$ platelets/µl with autologous platelet poor plasma. Light transmission during Adenosine 5'-diphosphate (ADP) – induced platelet aggregation was recorded on a four-channel aggregometer (Chrono-log Corp. Havertown, PA). Four minutes before stimulation with a sub-threshold concentration of ADP, the PRP was incubated during 3 min at 37 °C either with 75 µg/ml intact Mab 5H2 or 50 µg/ml of its F(ab')2 fragments or its Fab' fragments.

Statistical analysis

Inter-group comparison was performed with the Mann-Whitney U test and potential correlations were evaluated using the Spearman rank order test. P-values lower than 0.05 were considered significant.

Results

Selection and in vitro characterization of monoclonal antibodies reacting with hamster $\beta_2$-glycoprotein I

Murine monoclonal antibodies (Mabs) previously raised against human $\beta_2$-glycoprotein-I ($\beta_2$GPI) were selected on the basis of their cross-reactivity with hamster $\beta_2$GPI. Two Mabs, 5H2 and 11E8, strongly binding to hamster $\beta_2$GPI attached to phosphatidylserine, were selected. Mab 27A8, reacting with human $\beta_2$GPI only, was selected as negative control. Mab 5H2 had potent lupus anticoagulant (LA) activity in hamster plasma as documented by the concentration-dependent prolongation of the dilute prothrombin time (dPT). The dPT of hamster plasma spiked with 5H2 at 40 µg/ml was prolonged by a factor 1.50. Mab 11E8 did not possess LA activity. Mab 5H2 was therefore selected for the preparation of F(ab')2 and Fab' fragments; their affinity for human $\beta_2$GPI was similar to that of the intact antibody when tested in microtiter plates coated with $\beta_2$GPI. Both intact 5H2 and its F(ab')2 fragments induced hamster $\beta_2$GPI binding to phospholipids (75% phosphatidylcholine and 25% phosphatidylserine), which was not the case for Fab' fragments (Fig. 1).
Fig 1. Antibody induced binding of hamster β2GPI to phospholipids. Induction of β2-GPI binding to phospholipids (75% phosphatidylcholine and 25% phosphatidylserine) by intact 5H2 or its F(ab’)2 fragments compared to Fab’ fragments. Antibody-β2GPI complexes, attached to microtiter plate coated phospholipids, were measured with a rabbit anti-β2GPI antiserum coupled to peroxidase.

The bell-shaped relationship between the concentration of intact 5H2, or its F(ab’)2, and β2GPI binding to the phospholipid surface, suggests formation of bivalent antibody-β2GPI complexes on the phospholipid surface; at very high antibody concentration, competition
between monovalent and bivalent complexes causes a decrease in amount of β2GPI bound to phospholipids (Fig. 1). The concentration-dependent inhibition of intact 5H2-induced β2GPI binding to phospholipids by the 5H2 derived Fab’ fragments confirms the formation of bivalent complexes between 5H2 and hamster β2GPI (data not shown).

**Thrombogenicity of 5H2**

Both Mabs cross-reacting with hamster β2GPI, one with (5H2) and one without LA activity (11 E8), were injected intravenously at a dose ranging from 0 to 10 mg/kg prior to application of a controlled vessel injury. Mab 27A8, non-reactive with β2GPI, and buffer were selected as negative controls. 5H2 dose-dependently promoted thrombus formation, enhancing the median total intensity of transilluminated light, calculated as area under the curve (AUC), from 6.0 (median value, n=9) arbitrary light units (AU) in the controls treated with buffer to 65.0 AU in the 5H2 high dose group (10 mg/kg, n=6, p=0.007) with an ED50 around 1.1 mg/kg (median 24.5 AU, n=6) (fig. 2). The LA negative Mab 11E8 promoted thrombus formation only marginally (median AU at a dose of 10 mg/kg: 14.3, n=8, p=0.18, not shown). The influence of Mab 27A8 on thrombus development was negligible (median AU: 9.7, n=6, not shown).

In a second set of experiments, hamsters (n=8) treated with intact 5H2 at a dose of 3.3 mg/kg were compared with animals treated with 5H2 derived F(ab’)2 fragments at a dose of 2.2 mg/kg (n=7) and 4.5 mg/kg (n=8) and control animals (n=16). F(ab’)2 fragments promoted thrombus formation similarly to the intact antibody both at an equimolar dose (2.2 mg/kg) and a double equimolar dose (4.5 mg/kg) (fig 2b). The median AU for the control group and for the animals receiving intact 5H2 and its F(ab’)2 fragments at an equimolar dose and a double equimolar dose were 17.9, 55.8, 62.3 and 43.2 AU respectively. The differences between the treated groups and the control group were all statistically significant. No statistical differences were found between the 3 treated groups.

A last series of animal experiments revealed lack of thrombogenicity of the 5H2 derived Fab’ fragments administered at a dose of 2.2 mg/kg (median thrombus light intensity 3.3 AU [n=6] vs. 6.1 AU [n=9] in the control group, p=0.556; fig. 2c).
Fig. 2. Photochemically induced thrombosis of hamster carotid artery: (a) dose-dependent effect of intact 5H2 on cumulative thrombus formation over 40 min expressed as total intensity of transilluminated light; (b) comparison of thrombus light intensity in animals treated with intact 5H2 or its F(ab')2 fragments at the indicated doses; (c) influence of 5H2 Fab' fragments on thrombus formation (data are presented as median, interquartile range [IQR] and min-max range of area under curve; p-values for comparison with controls were calculated using Mann-Whitney U test);
The median antibody levels measured in the plasma collected just after completion of the experiments were 37 µg/ml for the group having received intact 5H2, and 15 and 35 µg/ml for the animals treated with the lower or higher F(ab’)2 dose. No significant correlation was found between the antibody or antibody fragment concentrations and the thrombus light intensity (Spearman rank order correlations: R=0.405, 0 and 0.309; p=0.320, 1 and 0.456, respectively). Immunohistochemical analysis of carotid artery thrombi showed that intact 5H2 (fig. 3) and its F(ab’)2 fragments (data not shown) were mainly found in association with platelets within the platelet-rich thrombus and were to a much lesser extent bound to vascular endothelium.

Fig. 3. Immunohistochemical evidence for the presence of intact 5H2 in the thrombus. Hematoxylin-eosin staining of control thrombus induced by prolonged exposure (4 min) to Xenon light (a) and thrombosis promoted by 10 mg/kg IV 5H2 (b). Immunohistochemical stainings of control thrombus (c) and of 5H2 promoted thrombus (d). Insert shows association of 5H2 with individual platelets inside the thrombus.
Since 5H2 and its F(ab’)2 fragments both promoted platelet-rich thrombus formation in vivo, we studied the effect of the intact Mab and its fragments on platelet aggregation in vitro using optical aggregometry. 5H2 by itself did not induce platelet aggregation, even when used at concentrations up to 200 µg/ml. However, when sub threshold concentrations of ADP, by themselves only inducing a first wave of aggregation, were added to PRP preincubated with 75 µg/ml 5H2, strong aggregation responses were observed (Fig 4). Equimolar concentrations of the F(ab’)2 fragments (50 µg/ml) also promoted ADP-induced aggregation whereas Fab’ fragments did not.

![Graph showing platelet aggregation](image)

Fig. 4. Effect of intact 5H2 and its fragments on platelet aggregation induced by low concentrations of ADP. Platelets were incubated for 4 min at 37 °C with 5H2 (75 µg/ml), its F(ab’)2 (50 µg/ml) or Fab’ fragments (50 µg/ml) or buffer and then stimulated with a subthreshold concentration of ADP (1.10 µM) causing by itself only a first wave of aggregation. Tracings representative of four different experiments are shown.
Discussion

The association between the presence of aPL and thrombosis affecting both veins and arteries is well established (1). In addition, prospective studies, showing that elevated aPL are a risk factor for future thrombosis, suggest that aPL may be involved in thrombogenesis (29, 30). More direct evidence for the thrombogenicity of aPL was provided by animal models employing vessel wall injury to induce thrombosis (ref. 31-35 and present study). Thus, after limited mechanical injury to the femoral vein in CD-1 mice, enhanced thrombosis was observed at the site of injury as well as slower thrombus disappearance after injection of immunoglobulin, affinity-purified aCL and even a monoclonal IgG aCL, all from patients with APS (31-33). Similar observations were reported after active immunization with human β2GPI (34). In this experimental setting, murine monoclonal aCL possessing LA properties were thrombogenic whereas an anti-β2GPI antibody without LA activity had no effect (35). These studies implicate aPL in venous thrombus formation but no direct support is available for the notion that aPL may be involved in arterial thrombosis.

Therefore, in the present study the impact of aPL was investigated in a model of carotid artery thrombosis in the hamster. This animal model complies with the concept of thrombosis as a “double hit” phenomenon. Very mild thrombosis is provoked by limited photochemically induced injury to the vessel wall (“first hit”). This injury affects the entire area of the irradiated vascular segment but is confined to the endothelium. In this model, factors promoting platelet activation (36) or coagulation (25) enhance thrombus formation (“second hit”). Mabs previously raised against human β2GPI (15) were selected on the basis of their cross-reactivity with hamster β2GPI and their LA and aCL properties in hamster plasma. We found that the LA and aCL positive Mab 5H2, which cross-reacts with hamster β2GPI, dose-dependently promoted thrombus formation (see fig 2a). This finding provides the first direct evidence that aPL may indeed be implicated in arterial thrombosis. Mab 27A8, non cross-reacting with hamster β2GPI and chosen as a negative control, had no clear effect. The thrombogenic effect of the aCL-positive but LA-negative anti-β2GPI Mab 11E8 was not significant, a finding that is in line with the clinical observation that a LA is more strongly associated with thrombosis than aCL (20, 21).
The availability of sufficient quantities of Mab 5H2 enabled us to prepare F(\(ab\'))\(_2\) and Fab’ fragments from this antibody and to evaluate whether bivalent hamster \(\beta_2\)GPI-antibody complex formation on phospholipid surfaces and possible Fc involvement constitutes the basis for the development of thrombotic complications. The F(\(ab\'))\(_2\) and Fab’ fragments of 5H2 had similar affinity for \(\beta_2\)GPI as the intact Mab (data not shown). The 5H2 derived F(\(ab\'))\(_2\) fragments and the intact Mab 5H2 promoted binding of hamster \(\beta_2\)GPI to phospholipid surfaces equally well (see fig 1). As shown previously with other LA positive anti \(\beta_2\)GPI Mabs, this binding was concentration-dependently inhibited by their Fab’ fragment that prevents bivalent \(\beta_2\)GPI-antibody complex formation (15).

An important and novel finding of this study is that F(\(ab\'))\(_2\) fragments derived from a LA positive Mab enhance arterial thrombosis *in vivo*. This somewhat unexpected finding strengthens our hypothesis that the thrombogenicity of aPL relies on cellular activation by surface bound bivalent antigen-antibody complexes, but weakens the suggested involvement of cellular Fc\(\gamma\)R receptors or the complement system (22, 23). Animal models of venous thrombosis have provided evidence for Fc-receptor independent thrombotic mechanisms (37). In venous thrombosis, bivalent \(\beta_2\)GPI-antibody complexes may reduce the anticoagulant effects of protein C and protein S by competition for the phospholipid surface on which they function (19). Immunohistochemical analysis of the arterial thrombus formed (see fig 3) localized 5H2 and its F(\(ab\'))\(_2\) fragments mainly to platelets in certain areas of the thrombus. This allows to propose the following scenario: following mild endothelial damage, a small platelet thrombus develops (first hit); the slightly activated platelets expose negatively charged phospholipid; this leads to patchy deposition of bivalent \(\beta_2\)GPI-antibody complexes; these complexes cause further platelet activation and thrombus growth (second hit). In contrast to a previous suggestion (38), binding to endothelial cells seems less involved. The possibility that bivalent \(\beta_2\)GPI-antibody complexes might promote platelet activation in an Fc-independent manner was tested in vitro. Intact 5H2 further stimulated platelet aggregation induced by sub threshold concentrations of ADP. At least part of this proaggregatory effect was Fc-independent since equimolar concentrations of F(\(ab\'))\(_2\) also potentiated ADP-induced aggregation whereas Fab’ fragments did not. Recent work by others further strengthens the concept of platelet activation by \(\beta_2\)GPI dimers. A chimeric recombinant protein consisting of two \(\beta_2\)GPI molecules linked together through the dimerization domain (apple4) of factor XI at the amino-terminal ends of \(\beta_2\)GPI domain I also has lupus anticoagulant properties (39).
and enhances thrombus formation when added to whole blood perfused over a collagen surface (40). How β2GPI dimers increase platelet deposition in vitro (40) and thrombus formation in vivo (this study), is subject to ongoing research. In conclusion, the present study has revealed that certain antiphospholipid antibodies enhance arterial thrombosis by forming bivalent β2GPI-antibody complexes with affinity for phospholipid, and that this prothrombotic action is largely Fc-independent.

Acknowledgements

This study was supported by a grant from the Flemish Fund for medical scientific research ‘Levenslijn 7.0032.98’ and ‘FWO G.0226.01’.

M. Jankowski was on leave of absence from the Jagiellonian University School of Medicine, Skawinska 8, 31-066 Krakow, Poland and was supported by a grant, BIL098/38, from a bilateral agreement between Flanders and Poland.


References


Thrombogenicity of β2-glycoprotein I-dependent antiphospholipid antibodies in a photochemically-induced thrombosis model in the hamster

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