Development of auto-antibodies towards β2-glycoprotein I in the antiphospholipid syndrome

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CHAPTER 5

Two different populations of domain I anti-β₂GPI antibodies; one inhibits and one stimulates *in vitro* thrombin generation

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ABSTRACT

Antiphospholipid antibodies are associated with thrombosis in vivo and with a prolonging clotting time in vitro. Beta2-Glycoprotein I (β₂GPI) is the major antigen in the antiphospholipid syndrome. This protein can exist in two conformations, a circular conformation present in plasma that can change into a fish-hook conformation. Only the fish-hook conformation is able to interact with anti-β₂GPI patient antibodies. Here we aim to elucidate the apparent paradox of anticoagulant antiphospholipid antibodies in vitro and their association with thrombosis in vivo.

To investigate the effect of β₂GPI and anti-β₂GPI antibodies on coagulation calibrated automated thrombography (TG-CAT) was used. We observed that β₂GPI in a fish-hook conformation but not in a circular conformation was able to inhibit coagulation when initiated with low tissue factor. Murine and human anti-β₂GPI antibodies, with affinity for β₂GPI regardless of its conformation decreased peak height and increased lag time for the TG, displaying the classic lupus anticoagulant activity in vitro. However, murine and human anti-domain I monoclonal antibodies with affinity only for fish-hook shaped β₂GPI, showed an increased peak height and a shortened lag time when TG was initiated with low TF concentrations (0.5 and 1pM TF).

Patient plasmas can contain two different populations of antibodies directed against domain I of β₂GPI. The two antibody populations differ in two respects, they recognize different epitopes on domain I of β₂GPI and they have a diametrically opposite effect on thrombin generation in vitro.
INTRODUCTION

The antiphospholipid syndrome (APS) is characterized by the persistent presence of circulating antiphospholipid antibodies in plasma of patients with a history of thrombotic events and/or obstetrical complications. \( \beta_2 \)-Glycoprotein I (\( \beta_2 \)GPI) is regarded as the major antigen in this syndrome. This protein is a 43 kDa glycoprotein consisting of five complement control protein domains connected by 3 or 4 amino acids that form flexible linkers. Domain I contains an epitope recognized by a population of autoantibodies that highly correlates with the clinical manifestations of APS, whereas the fifth domain contains a patch of positively charged amino acids and a hydrophobic insertion loop together harboring the phospholipid binding site. \( \beta_2 \)GPI circulates in blood in a circular conformation and upon binding to negatively charged surfaces with its positively charged \( V^\beta \) domain, the conformation of \( \beta_2 \)GPI is changed into a fishhook-like shape. When the circular conformation of \( \beta_2 \)GPI changes into a fish-hook shaped conformation a cryptic site within domain I becomes exposed on the outside of the protein. \( \beta_2 \)GPI can now be recognized by thrombosis-related anti-domain I antibodies. Recently, an interesting theory was proposed in the etiology of auto-antibodies against \( \beta_2 \)GPI. It was hypothesized that patient antibodies are induced due to continuous exposure to fish-hook conformation of \( \beta_2 \)GPI in the circulation which can be caused by the interaction of \( \beta_2 \)GPI with e.g. bacterial proteins.

An informative way to measure effects of antiphospholipid antibodies on coagulation is thrombin generation. Thrombin generation can be measured by the conversion of a thrombin-sensitive fluorescent probe by thrombin generated during ongoing coagulation. Hemker et al. designed a method by which thrombin generation can be quantified, adjusted for substrate depletion and inner-filter effect by adding a calibrator in fixed dose (alpha-2M-thrombin). Calibrated automated thrombography (TG-CAT) has been described by several groups as a method to detect antiphospholipid antibodies with lupus anticoagulant activity. Anticoagulant antiphospholipid antibodies prolong lag times and decrease peak heights and sometimes the endogenous thrombin potential (ETP).

The characterization of the APS is based on a paradox; clinically the disease is characterized by thrombotic events, while it is diagnosed by a phospholipid dependent delay in the coagulation. The experiments described in this manuscript tried to provide an explanation for this paradox. We have found a subpopulation of anti-domain I anti-\( \beta_2 \)GPI antibodies that under certain conditions displays a prothrombotic phenotype in vitro.
RESULTS

Recently, we published that \( \beta_2 \text{GPI} \) can exist in two conformation, a circular plasma conformation and fish-hook like conformation in complex with the auto-antibodies \( \text{(10)} \). We have identified two human and two murine monoclonal antibodies directed against domain I. The monoclonal antibody (moAb) \( \text{P1-117 (human)} \) and \( 4F_3 \) (mouse) recognize only fish-hook shaped \( \beta_2 \text{GPI} \), \( 3B_7 \) (mouse) and \( \text{P2-6 (human)} \) recognize both circular and fish-hook shaped \( \beta_2 \text{GPI} \). This indicates that \( 4F_3 \) and \( \text{P1-117} \) recognize a cryptic epitope in \( \beta_2 \text{GPI} \) that becomes accessible when \( \beta_2 \text{GPI} \) changes into a fish-hook shaped conformation (\text{table 1}). In our laboratory we observed that antibodies recognizing only the fish-hook shaped conformation of \( \beta_2 \text{GPI} \) does not display lupus anticoagulant activity \( \text{(figure 1a and 1b).} \) \( \text{P2-6} \) remains to be tested.

In order to investigate the effect of the different monoclonal antibodies on coagulation \textit{in vitro}, the TG-CAT-method to measure thrombin generation was applied. The different monoclonal antibodies \( \text{\( 3B_7, 4F_3, P2-6 \) and \( P1-117 \)} \) were diluted in plasma to a final concentration of \( \text{100 \ \mu g/mL} \). When TG was initiated with a high TF concentration \( (5 \ \text{pM} \ \text{TF}) \) no effect of the antibodies was observed on either peak height \( \text{(PH)} \), lag time \( \text{(LT)} \) or endogenous thrombin potential \( \text{(ETP)} \) \( \text{(data not shown).} \) When TG was initiated with \( 0.5 \ \text{pM} \ \text{TF} \) we observed for \( \text{moAb 3B}_7 \) \( \text{(recognizing both conformations of beta2GPI)} \) an increased lag time and decreased peak height compared to normal pooled plasma TG \( \text{(LT: 11.8 vs. 9.5 minutes p<0.05, PH: 144.2 vs. 221 nM thrombin p<0.05)} \) indicating normal lupus anticoagulant activity. A similar pattern we observed for \( P2-6, \text{(LT: 10.5 vs. 9.5 minutes p<0.05, PH: 143.1 vs. 221 nM thrombin p<0.05).} \) However, in the presence of \( \text{moAb P1-117 and 4F}_3 \) \( \text{(both recognizing only fish-hook shaped beta2GPI)} \) a shorter lag time and increased peak height compared to normal pool plasma was observed \( \text{(LT: 9.0 (P1-117 p<0.05 vs NP) vs. 7.8 (4F}_3 \text{ p<0.05 vs NP) vs. 9.5 (NP), PH: 295.0 (P1-117 p<0.05 vs NP) vs. 316.3 (4F}_3 \text{ p<0.05 vs NP) vs. 221 (NP).} \) This indicates that both \( \text{P1-117 and 4F}_3 \) promote thrombin generation \( \text{(figure 2).} \)

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Origin</th>
<th>Type</th>
<th>Circular ( \beta_2 \text{GPI} )</th>
<th>Fish-hook ( \beta_2 \text{GPI} )</th>
<th>Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 3B_7 )</td>
<td>Mouse</td>
<td>IgG</td>
<td>+</td>
<td>+</td>
<td>DI</td>
</tr>
<tr>
<td>( 4F_3 )</td>
<td>Mouse</td>
<td>IgG</td>
<td>-</td>
<td>+</td>
<td>DI</td>
</tr>
<tr>
<td>( \text{P1-117} )</td>
<td>Human</td>
<td>IgG</td>
<td>-</td>
<td>+</td>
<td>DI</td>
</tr>
<tr>
<td>( \text{P2-6} )</td>
<td>Human</td>
<td>IgG</td>
<td>+</td>
<td>+</td>
<td>DI</td>
</tr>
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</table>
For the moAb 3B7 the LAC was neutralized in the presence of an excess of phospholipids (FIGURE 3). For both the moAbs 4F3 and P1-117 an increasing concentration of phospholipids did not influence the lag time (FIGURE 3A). It further increased peak height, although at 32 µM we observed a decrease for 4F3 (FIGURE 3B). As for the LAC the ETP remained uninfluenced by an excess of phospholipids (FIGURE 3C).

**FIGURE 1.** Lupus anticoagulant activity of the monoclonal anti-β₂GPI antibodies. (A) LAC ratio for the aPTT. (B) LAC ratio for the dRVVT. Dotted line is set at 1.15 and is the threshold for LAC, based on 99th percentile of 40 healthy individuals.

**FIGURE 2.** Thrombin generation of plasma spiked with anti-β₂GPI antibodies (P1-117, P2-6, 4F3 and 3B7), initiated by 0.5 pM tissue factor and 4 µM phospholipids. The antibody recognizing both conformations of β₂GPI (3B7, P2-6) showed delayed and less thrombin generation, whereas the antibodies recognizing a cryptic epitope in β₂GPI (P1-117 and 4F3) showed faster and increased thrombin generation.
The differential effects of the moAbs on thrombin generation appeared to be related to the conformation of $\beta_2$GPI. Therefore the effect of both conformations on thrombin generation was tested. Plasma purified circular $\beta_2$GPI did not affect thrombin generation when initiated with any concentration of TF (FIGURE 4A). Additionally, fish-hook shaped $\beta_2$GPI inhibited thrombin generation (FIGURE 4C, 4D, 4E). FIGURE 4C shows that an increase in TF abrogated the inhibitory effect of fish-hook shaped $\beta_2$GPI on thrombin generation. By initiating TG with 0.5 pM TF a dose dependent effect of open $\beta_2$GPI is observed (FIGURE 4B).

**FIGURE 3.** Various concentrations of phospholipids were incubated with plasma spiked with anti-$\beta_2$GPI moAbs. 3B7 recognizes both conformations of $\beta_2$GPI; P1-117 and 4F3 only the open conformation. (A) Lagtime was increased with increased phospholipid concentration, for moAb 3B7 the increase in lag time was neutralized with higher phospholipid concentration. Lag time for moAb P1-117 and 4F3 remained uninfluenced. (B) Peak height remained stable with increasing phospholipid concentration for NP and in the presence of 3B7, peak height varied for 4F3 and increased for P1-117. (C) Endogenous thrombin potential slightly decreased in the presence of higher phospholipid concentrations, a similar effect is observed for 3B7. No overall effect was observed for plasma spiked with either P1-117 or 4F3.
The effects of P1-117 and 4F3 on thrombin generation were only observed at low tissue factor concentration, suggesting that the antibodies modulate one of the feedback amplification loops of coagulation. It is known from the literature that β₂GPI binds both to thrombin and factor XI\textsuperscript{16,17}. Therefore we have studied the effects of β₂GPI with or without the antibodies on the conversion of factor XI to XIa by thrombin. To do so, we incubated the different MoAb with β₂GPI and mixed this with FXI followed by activation with thrombin. For both the moAbs P1-117 and 4F3 we observed an enhanced FXI activation, whereas no effect was observed for Moab 3B7 (FIGURE 5). The antibodies in the absence of β₂GPI did not influence FXI activation.

**FIGURE 4.** Thrombin generation in the presence of β₂GPI. TG was initiated with tissue factor in NPP spiked with either closed or open β₂GPI in the presence of 4 μM phospholipids. (A) TG initiated with 5 and 1 pM TF in the presence of 50 μg/ml circular β₂GPI showed no effect. (B) Dose dependent effect on thrombin generation of β₂GPI bound to phospholipids is observed when TG is initiated with low TF. Values are average ± SD. TG initiated with either (C) 5, (D) 1 or (E) 0.5 pM TF with and without 50 μg/ml open β₂GPI. No effect of open β₂GPI is observed on TG when this is initiated with high TF. However, when the TF concentration declines a decrease in thrombin generation is observed.
DISCUSSION

Publications about the role of $\beta_2$GPI in coagulation are diverged and no, pro- and anticoagulant effects of $\beta_2$GPI have been claimed\textsuperscript{18,19,20}. The association between the presence of anti-$\beta_2$GPI antibodies and thrombosis is well established in animals\textsuperscript{21,22,23} and man\textsuperscript{24,25}. Although the lupus anticoagulant correlates stronger with the risk for thrombosis in human, a weak correlation is also found for anti-$\beta_2$GPI antibodies, especially for the subpopulation of anti-$\beta_2$GPI antibodies that are directed to domain I of this protein\textsuperscript{26,27}. The observation that $\beta_2$GPI can exist in two different conformations in combination with a panel of human and murine monoclonal antibodies prompted us to more closely analyze the complicated interaction of $\beta_2$GPI with hemostasis. To do so, we have used thrombin generation assays to analyze the hemostatic balance more subtle. We showed that that plasma $\beta_2$GPI itself did not influence the thrombin generation time. Auto-antibodies that recognize domain I of $\beta_2$GPI irrespectively of its conformation inhibited thrombin generation, expressing a classic lupus anticoagulant. Interestingly, auto-antibodies against $\beta_2$GPI that recognize domain I only when $\beta_2$GPI is in its open conformation decreased thrombin generation when coagulation is started with low tissue factor concentrations, expressing what one could call a lupus ‘procoagulant’ activity. As expected, the effects of the antibodies with a lupus anticoagulant activity could be neutralized when the phospholipid concentrations in the assays is increased, mimicking the lupus anticoagulant confirmation assay. In

FIGURE 6. Effect of anti-$\beta_2$GPI in thrombin mediated FXI activation. MoAbs $\beta_2$GPI was preincubated with the moAbs and mixed with FXI. FXI (30nM) was activated by thrombin (10nM) for 5 minutes at 37°C. Moab anti-$\beta_2$GPI P1-117 and 4F3 stimulate the activation of FXI by thrombin.
contrast, the effects of the antibodies with lupus procoagulant activity strengthened when the phospholipid concentrations are increased.

It has been shown that $\beta_2$GPI binds to both factor XI and thrombin$^{16,17}$. These observations, in combination with the observation that the lupus procoagulant effect is only observed at low tissue factor concentrations, prompted us to study the effects of the anti-$\beta_2$GPI antibodies on the thrombin mediated factor XI activation, an amplification loop in coagulation that is not detected with classic lupus anticoagulant assays. We could show that the antibodies that only recognize $\beta_2$GPI in its open conformation accelerated the conversion of factor XI by thrombin, thereby explaining the observed lupus procoagulant effect of these antibodies. This immediately explains why this effect of anti-$\beta_2$GPI antibodies on coagulation in the classic lupus anticoagulant assays remained unobserved before.

The relevance of this newly identified subpopulation of auto-antibodies against $\beta_2$GPI for the pathophysiology of the antiphospholipid syndrome remains to be established. Nevertheless, the observations made in this study showed that there is an urgent need to further characterize the different subpopulations of auto-antibodies against domain I of $\beta_2$GPI. It has been shown extensively that auto-antibodies against domain I of $\beta_2$GPI correlate much better with the clinical manifestations that characterize APS. We have shown here that there are at least two different antibody populations that recognize domain I with completely different characteristics. Our next step is to develop assays specific for both antibody populations and correlate their presence with the clinical manifestations of APS.

**MATERIALS AND METHODS**

**Proteins**

Plasma $\beta_2$GPI was isolated from fresh citrated human plasma from 3 healthy donors as previously described$^{18}$. Purity of $\beta_2$GPI was determined with sodium dodecyl sulfate (SDS)–polyacrylamide (PAA) gel electrophoresis (GE Healthcare). Purified plasma $\beta_2$GPI showed a single band with a molecular mass of approximately 43 kDa under non-reducing conditions. The concentration of the protein was determined with the bicinchoninic acid protein assay (Thermo Fisher Scientific LSR). This purified $\beta_2$GPI is in the circular plasma conformation, this is switched to the fish-hook conformation as previously described$^{10}$.

Recombinant proteins. Human $\beta_2$GPI cDNA (kindly provided by Dr. T. Kristensen from the university of Aarhus, Denmark) was used to construct full length recombinant $\beta_2$GPI. cDNA was subcloned into a PCR-Blunt II-TOPO vector (Invitrogen) and the
protein was constructed with a set of two primers with BamHI and NotI restrictions sites; 5’ GGATCCGGACGGACCTGTCCCAAGCC 3’ and 5’ GCGGCCGCTTAGCATGCGCTTTACATCGG 3’. The PCR product was cloned into a PCR-Blunt II-TOPO vector, and sequence analysis was performed to confirm the sequence of β2GPI. From this vector, the PCR product was subcloned into the expression vector HisN-Tev (Promega). Individual domain I and domain V were cloned as previously described10.

**Protein expression and purification**

Recombinant β2GPI and the separate domains were expressed in HEK293E cells and collected from a nickel Sepharose column with an elution buffer (25 mM tris(hydroxymethyl)aminomethane, 500 mM NaCl, 500 mM imidazole, pH 8.2). To increase purity, proteins were loaded to a Hi-load Superdex 200 XK26 column (GE Healthcare). β2GPI and the separate domains were > 95% pure as checked on a 4-15% SDS-PAA gel (GE Healthcare).

Normal pooled plasma. Normal pooled plasma (NPP) was obtained from more than 200 healthy individuals. Blood was drawn in 3.2% citrate and spun down twice at 2000 x g. All healthy individuals gave written informed consent and the Ethical Review Board of the Academic Medical Center Amsterdam, the Netherlands.

**Antibodies**

Two monoclonal mouse-anti-human β2GPI antibodies: 3B7 and 4F3 were purified from hybridoma medium using protein G-Sepharose. P1-117 was obtained from an APS patient as previously described29. The antibodies were >99% pure as checked on a 4-15% SDS-PAA gel (GE Healthcare).

**Thrombin generation assay**

Thrombin generation was triggered by different concentrations of tissue factor (TF). To quantify thrombin generation the TG-CAT-method was used as previously described13. In short 80 µL of test plasma and 20 µL of activator (TF or XIa with 4 µM phospholipid (PC/PS/PE, 60/20/20)) was added to a round-bottom ELISA plate (Immunonlon2HB, Thermolab System, Helsinki, Finland). The plate containing the test plasma and trigger reagent was heated at 37°C for 10 minutes. Thrombin generation was initiated by adding 20 µL Z-GGR-AMC (2.5 mM) and CaCl₂ (100 mM). Calibration was performed by adding thrombin calibrator (final thrombin activity of 100 nM) to the test sample. The obtained thrombogram provided the lag
time (minutes), endogenous thrombin potential (ETP, nM.minutes) and thrombin peak height (nM).

Coagulation assays

The LAC activity of anti-β₂GPI moAbs was investigated in normal pooled plasma. Antibodies were preincubated in NPP for 2 minutes at 37°C. A dRVVT was performed according to the protocol of the manufacturer (LA Screen; Life Diagnostics, Sydney, Australia) and confirmed with LA-2. APTT was determined with a LAC-sensitive PTT-LA assay (Diagnostica Stago, Asnières, France) the screen was confirmed with Actin FS a lupus anticoagulant independent reagent (Siemens Healthcare Diagnostics, Marburg; Germany).
REFERENCES


