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

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CHAPTER 7

Auto-antibodies against $\beta_2$ GPI: Etiology and mechanism of action

General discussion and summary

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THE ANTIPHOSPHOLIPID SYNDROME

The antiphospholipid syndrome (APS) is an autoimmune disease characterized by the persistent presence of antiphospholipid antibodies (aPL) in plasma of patients with thrombotic events and/or recurrent fetal loss. Although the name of the syndrome suggests otherwise, phospholipids are not the antigen but rather proteins with affinity for anionic phospholipids. The presence of aPL antibodies can be detected in three ways: with prolongation of clotting assays, known as lupus anticoagulant (LAC), and with two different ELISA set-ups with either cardiolipin or \( \beta_2 \)-Glycoprotein I (\( \beta_2 \)GPI) as antigens. Although the outcome of these tests can vary strongly, auto-antibodies towards \( \beta_2 \)GPI should be the major determinant in all three assays. As the definition of APS stated that only persistently present aPL are considered relevant, the tests should be positive twice, 12 weeks apart. aPL are often present during infections and by measuring at two separate occasions falsely positive results for these tests due to infections can be excluded and APS can be properly diagnosed.

\( \beta_2 \)GPI: THE MAIN ANTIGEN

\( \beta_2 \)GPI is considered to be the major antigen in the antiphospholipid syndrome. \( \beta_2 \)GPI is a plasma protein belonging to the complement control protein (CCP) family and it consists of five separate domains. Domain I to domain IV contain each ~60 amino acids folded together by two disulfide bridges. Domain V is slightly larger and contains an extra disulfide bridge. The extra amino acids constitute a phospholipid binding site. \( \beta_2 \)GPI is highly glycosylated and its glycans comprise approximately of 20% the weight of the protein. Recently, we showed that \( \beta_2 \)GPI can exist in two conformations (Chapter 2 of this thesis). In plasma, \( \beta_2 \)GPI is in a closed conformation, which cannot be recognized aPL. After interaction with anionic phospholipids, the conformation is forced to an open fish-hook shaped structure, similar to the structure revealed by crystallography. This fish-hook conformation can be recognized by aPL. The clinical relevant epitope for the auto-antibodies is located within the first domain of \( \beta_2 \)GPI around amino acids Arg39-Arg43. Interaction with phospholipids or a hydrophilic ELISA tray switches the conformation of \( \beta_2 \)GPI.

ORIGIN OF ANTI-\( \beta_2 \)GPI ANTIBODIES

Several studies have successfully linked the presence of anticardiolipin antibodies to a history of infections. However, when these patient groups were tested for the presence of anti-\( \beta_2 \)GPI antibodies no correlation was found. Antibodies are generated by
the immune system to neutralize non-self substances in your body. The antibody recognizes a unique epitope of the foreign material and by binding to this epitope the antibody marks the pathogen and identifies it for neutralization cells of the immune system. Occasionally the immune system does not function properly and antibodies towards self proteins are developed. The general consensus is that the auto-antibodies are induced by an infection. There are two theories that try to explain why antibodies towards self proteins are developed (a) molecular mimicry and (b) the infectious agent as adjuvant: the exposure of cryptic epitopes normally not exposed to the circulation. Until recently most evidence about the origin of anti-\(\beta_2\)GPI antibodies pointed in the direction of molecular mimicry. However, the discovery that \(\beta_2\)GPI can exist in two completely different conformations draw our attention to the hypothesis of an infection as adjuvant. The next part presents data published in the search to the origin of autoantibodies against \(\beta_2\)GPI.

**MOLECULAR MIMICRY**

Infectious agents can contain epitopes that are similar to epitopes of self-proteins. An immune response towards the infectious agent may additionally result in a response towards the self-proteins. Sequence similarities between foreign and self proteins can be sufficient to induce a loss of immune tolerance resulting in the formation of autoantibodies. The group of Shoenfeld has shown homology between the peptide TLRVYK in domain III of \(\beta_2\)GPI and various microbial agents\(^{13}\). Additionally, it was shown that the presence of antibodies against this peptide in mice resulted in fetal resorption. The value of these observations for the human situation is questionable, since there are no indications that antibodies against domain III correlate with increased thrombotic manifestations and their presence only weakly correlates with recurrent spontaneous abortions in patients with APS\(^{14}\). It should also be considered that the third domain of \(\beta_2\)GPI is highly glycosylated, making peptide sequences in domain III unavailable for antibody recognition. In another study, Gharavi et al.\(^{15}\) injected mice with a peptide derived from cytomegalovirus with homology to an amino acid sequence present in domain V of \(\beta_2\)GPI. They found the induction of IgM antibodies directed against \(\beta_2\)GPI with functional properties comparable as found in APS. However, these antibodies were not observed in patients with acute cytomegalovirus infections\(^{16}\). In a third study, Krause et al.\(^{17}\) identified cross reactivity between antibodies against the cell wall of Saccharomyces cerevisiae and \(\beta_2\)GPI in patients with APS. However, the presence of these antibodies was not associated with any specific clinical manifestations of APS. In conclusion, it seems possible to induce auto-antibodies towards \(\beta_2\)GPI with peptides derived from infectious agents in mice, but there are no indications that the same auto-antibodies can be found in patients...
with APS. The lack of a correlation of these induced antibodies with thrombosis and fetal loss suggested to us that molecular mimicry is not a favorable theory to explain the etiology of anti-β₂GPI antibodies.

**Neo epitope of β₂GPI**

A more likely mechanism for the development of antibodies towards β₂GPI is in our opinion the neo-epitope theory. This theory claims that by altering the conformation of a self-protein, the proteins are no longer recognized as self and antibodies can develop towards the newly exposed epitopes. The conformational switch we have observed for β₂GPI fits with this theory. This conformational change of β₂GPI can be induced by various factors. In chapter 3 of this thesis we report that a cell membrane protein of *S. pyogenes*, protein H, is capable of switching the conformation of β₂GPI. β₂GPI is not the only protein in the circulation that can be the subject of substantial conformation changes (ref factor H). The question arises why, compared to other plasma proteins, auto-antibodies are formed against β₂GPI so often. A possible explanation is that the exposure of the neo-epitopes in β₂GPI occurs more often than in other plasma proteins. Theoretically the conformational switch in β₂GPI can be facilitated in different ways via: (a) intrinsic (by the protein itself), (b) endogenous and (c) exogenous factors.

**Table 1: Presence of anti-β₂GPI antibodies during an infection**

<table>
<thead>
<tr>
<th>Disease</th>
<th>serotype</th>
<th>ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Various infections</td>
<td>IgG</td>
<td>27</td>
</tr>
<tr>
<td>Acute syphilitic posterior placoid chorioretinitis</td>
<td>IgM</td>
<td>28</td>
</tr>
<tr>
<td>Inflammatory bowel disease</td>
<td>IgG</td>
<td>29</td>
</tr>
<tr>
<td>Macrovascular disease</td>
<td>IgA, IgM</td>
<td>30</td>
</tr>
<tr>
<td>Vasculitis in combination with infection</td>
<td>IgG, IgM</td>
<td>31</td>
</tr>
<tr>
<td>Leptospirosis, syphilis, kala-azar</td>
<td>IgG, IgM</td>
<td>32</td>
</tr>
<tr>
<td>Varicella chicken pox</td>
<td>IgM</td>
<td>33</td>
</tr>
<tr>
<td>HIV</td>
<td>IgG, IgM, IgA</td>
<td>34</td>
</tr>
<tr>
<td>Hepatitis B</td>
<td>IgG, IgM</td>
<td>35</td>
</tr>
<tr>
<td><em>E. coli, Proteus sp. and Klebsiella sp</em></td>
<td>IgG</td>
<td>36</td>
</tr>
<tr>
<td><em>S. pyogenes</em></td>
<td>IgG, IgM</td>
<td>37</td>
</tr>
</tbody>
</table>
**Intrinsic**

$\beta_2$GPI is a protein that shows a certain variation for both the coding sequence as well as the extent of glycosylation. These might be of influence on the ability to change the conformation of the protein. There are three well studied polymorphisms in the coding region of $\beta_2$GPI leading to amino acid substitutions: Val247Leu, Cys306Gly and Trp316Ser with respective frequencies between 0.30-0.50, 0.00-0.04 and 0.00-0.06, depending on the ancestry\(^{18,19}\). Although several publications claim a correlation between polymorphism Val247Leu and anti-$\beta_2$GPI antibodies, the overall consensus is that a correlation between auto-antibody formation and polymorphisms in $\beta_2$GPI is lacking\(^{20,21}\).

$\beta_2$GPI is a highly glycosylated protein with 4 N-linked glycan sites. Three glycosylation sites are located within Domain III at amino acids 143, 164 and 174 and the fourth site is present at amino acid 234 in domain IV. The glycans of the amino acid 164, 174, and 234 are directed to the inside of the fish hook according to the crystal structure\(^{5,6}\), whereas the glycan attached to Asn143 is located to the outside the protein. A hypothesis has been formulated that this glucose blocks the Arg39-Arg43 epitope\(^{11}\). This hypothesis has been developed further by Kondo et al. 2009 who showed that some APS patients have $\beta$GPI molecules with a reduced number of sialic acids in the glycan structure at Asn143\(^{22}\). They suggest that the alteration of the glycan alters the electrostatic properties leading to an instable conformation of $\beta_2$GPI and exposure of the cryptic Arg39-Arg43 site. Additionally, it has been suggested that advanced glycosylation at certain lysine residues in $\beta_2$GPI can interfere with its conformation thereby triggering dendritic cells. This alteration of conformation might also trigger auto-antibody formation\(^{23}\). All these findings are the result of in vitro biochemical research and only confirmed in very small selected patient groups. To establish whether changes in glycosylation of $\beta_2$GPI play a role in the development of auto-antibodies, these findings need to be confirmed in larger patient populations.

**Endogenous**

The conformation of $\beta_2$GPI is altered after binding to phospholipids in such a way that after binding it is possible to interact with the auto-antibodies\(^{11}\). Additionally, immunization of mice with $\beta_2$GPI bound to phospholipids leads to auto-antibody development towards $\beta_2$GPI\(^{24}\). This suggests that the presence of phospholipid surfaces could play a role in the exposure of neo-epitopes and anti-$\beta_2$GPI development. Indeed, Yamaguchi et al. showed that excessive exposure of anionic surfaces maintained the auto-antibody response to $\beta_2$GPI in APS patients\(^{25}\). These studies suggest that the etiology of anti-$\beta_2$GPI antibodies lies in interaction of $\beta_2$GPI with an anionic phospholipid surface. In this respect it is of interest that $\beta_2$GPI is involved in the clearance of apoptotic bodies and anionic microparticles (refs). Extensive apoptosis
can be a trigger to form auto-antibodies against $\beta_2$GPI.

**Exogenous**

The conformation of $\beta_2$GPI can also be altered by interaction with proteins of pathogens, as we have shown for protein H of *S. pyogenes* (Chapter 3). Despite the lack of larger studies on the presence of anti-$\beta_2$GPI antibodies in patient populations suffering from infections, there are numerous case reports on the presence of anti-$\beta_2$GPI antibodies after an infection (Table 1). The lack of studies on a correlation between infection and anti-$\beta_2$GPI antibodies is not surprising given that the frequency of anti-$\beta_2$GPI antibodies is low in the healthy population.\(^{26}\)

Although we cannot identify a single cause for the etiology of anti-$\beta_2$GPI antibodies, we hypothesize that the common denominator for the auto-antibody formation is the switch in conformation of the protein from a closed circular to an open fish hook conformation revealing the cryptic epitope in domain I. This open structure triggers the formation of antibodies.

It has been convincingly established that the permanent presence of auto-antibodies towards $\beta_2$GPI correlate with an increased risk of thrombus formation. However, these life-threatening clinical manifestations might be an unwanted side effect of an advantageous role that these auto-antibodies might have in innate immunity. There is *in vitro* evidence that the antibodies support the scavenging of LPS and the clearance of apoptotic bodies. The induction of autoantibodies towards $\beta_2$GPI during *e.g.* an infection might possibly help the innate immune system in its defense against infectious agents. As these antibodies are often reported in patients as response to different infectious diseases, they might help $\beta_2$GPI in fighting the infection, by maintaining $\beta_2$GPI in its open conformation, the conformation in which it can function as a scavenger for cellular debris and bacterial products.\(^{38}\) The presence of anti-$\beta_2$GPI antibodies might increase the efficiency of these processes.\(^{39-40}\) It has been suggested recently that a group of plasma proteins characterized by a positive charge and unclear function, like $\beta_2$GPI and PF4, have a role in the innate immunity by clearing undesired proteins from the circulation.\(^{41}\) Indeed recently it was found that $\beta_2$GPI in its open conformation can bind complement factor C3 and mediates cleavage of this complement factor.\(^{42}\)

**Auto-antibodies against $\beta_2$GPI in leprosy patients**

Auto-antibodies towards $\beta_2$GPI are an important characteristic to identify patients with APS. As SLE is very often the underlying disease in patients with APS, there
is a high incidence of these antibodies in patients with SLE. However, there is also one other patient population with a high incidence of these auto-antibodies: patients with leprosy. Leprosy is a chronic infection caused by mycobacterium leprae and the symptoms are dependent on the hosts’ response to the infection, however thrombotic and pregnancy complications are lacking. Approximately, 50% of these patients develop anti-\(\beta_2\)GPI IgM\(^{43,44}\). These patients also frequently develop anticardiolipin antibodies and LAC. The antibodies towards \(\beta_2\)GPI are mainly directed against domain V\(^{45}\) and, as in the human situation, isolated auto-antibodies from these patients do not display thrombotic effects in an \textit{in vivo} mouse model\(^{46}\). This is in contrast to the anti-\(\beta_2\)GPI antibodies isolated from APS patients which are mainly directed against domain I. The reason why these antibodies arise patient with leprosy and why they are directed specifically towards the fifth domain of \(\beta_2\)GPI remains unclear.

The pathogenic mechanism behind the anti-\(\beta_2\)GPI antibodies is properly multifactorial. The antibodies can activate endothelial cells, monocytes (See chapter 1 for a complete overview). However the study of Arad \textit{et al.} shows that minimal levels of anti-\(\beta_2\)GPI antibodies are required with a minimum of incubation time for the thrombotic effect\(^{53}\). This points to a direct interaction of \(\beta_2\)GPI/anti-\(\beta_2\)GPI complex for thrombus formation. We have shown (Chapter 5) that \(\beta_2\)GPI has an anticoagulant function in thrombus formation. When \(\beta_2\)GPI is bound to phospholipids it shows anticoagulant effect by the inhibiting the activation of FXI to FXIa. With the addition of anti-\(\beta_2\)GPI antibodies recognizing a cryptic epitope in \(\beta_2\)GPI, this anticoagulant effect turns over to a procoagulant effect. We observed faster and increased thrombin formation and hypothesize this to be due to release of FXI of \(\beta_2\)GPI due to anti-\(\beta_2\)GPI antibodies.

**CONSEQUENCES OF ANTI-\(\beta_2\)GPI ANTIBODIES**

Anti-\(\beta_2\)GPI antibodies prolong the coagulation time \textit{in vitro} while \textit{in vivo} they are a surrogate biomarker for a prothrombotic state. Antibodies directed against \(\beta_2\)GPI strongly increase the affinity of \(\beta_2\)GPI for phospholipids\(^{47}\), leading to competition with coagulation factor for phospholipids in coagulation assays \textit{in vitro}. However, \(\beta_2\)GPI auto-antibodies potentiate thrombus formation \textit{in vivo}. The mechanism by which these auto-antibodies induce a procoagulant state remains unknown. Anti-\(\beta_2\)GPI antibodies by themselves do not cause thrombosis; they enhance a thrombotic response when thrombus formation is triggered by e.g. vascular damage\(^{48}\).

Several population studies have shown that the presence of antibodies towards \(\beta_2\)GPI are correlated with an increased risk for thrombosis\(^{49,50,55}\). Auto-antibodies against \(\beta_2\)GPI consist of a heterogeneous population of antibodies and it has been shown that
antibodies towards the epitope of amino acids Arg39-Arg43 in domain I correlate much better with the risk for thrombosis than the total population of auto-antibodies against β2GPI in a population of APS patients42. Studies have shown that either total IgG from APS patients, moAb anti-β2GPI antibodies or human purified anti-β2GPI antibodies when injected into mice lead to a stronger prothrombotic response in mice that were subjected to a thrombosis model43-55.

The pathogenic mechanism behind the anti-β2GPI antibodies is probably multi-factorial. The antibodies can activate endothelial cells and monocytes (See chapter 1 for a complete overview). However the study of Arad et al. shows that minimal levels of anti-β2GPI antibodies are required with a minimum of incubation time for the thrombotic effect53. This points to a direct interaction of β2GPI/anti-β2GPI complex for thrombus formation. We have shown that anti-β2GPI antibodies recognizing a cryptic epitope have prothrombotic properties in vitro in contrast to antibodies recognizing other epitopes of β2GPI (Chapter 3).

**PERSISTENT PRESENCE OF ANTI-β2GPI ANTIBODIES**

There are numerous publications reporting the presence of anti-β2GPI antibodies in plasmas of patients with infections or other diseases. It is unclear from these publications whether the presence of these antibodies is transient of persistent. The general consensus is that these infection-related antibodies are transiently present but no studies have been published showing that these antibodies disappear after the infections. It is completely unclear why and when auto-antibodies against β2GPI become persistently present.

Genetic variation in the coding region of the protein does not correlate with the presence of antiphospholipid antibodies20-21. There is no scientific evidence that APS is a genetic disorder; the disease cannot be inherited from parents to children. However, there are indications that it can cluster in families indicating a genetic predisposition. APS is often observed together in association with other autoimmune diseases, the most important one being systemic lupus erythematosus (SLE). For SLE, a genome wide association study has been performed studying 317,501 single-nucleotide polymorphisms (SNP). Eight genomic regions have been identified to be associated with SLE56. Two of these SNPs were also found to be associated with APS: STAT4 and BLK with Odds ratios just above 257. As expected for APS or autoimmune diseases in general there is a suggestion of an underlying genetic component although this cannot fully explain the origin of the disease.
TREATMENT OF APS

APS is a rare disease and only a few small studies have been published comparing different treatments. Treatment of APS patients is rather eminence-based than evidence-based. There is now consensus on the treatment of thrombotic complications in APS patients. In the past, the patients were treated with high intensity vitamin K-antagonists, however, the recent guidelines suggest the same intensity as patients with thrombosis due to other causes. The controversy is how long the treatment should be and whether patients with antiphospholipid antibodies but without an event should receive prophylactic treatment. Currently, new anticoagulant medication is developed that specifically targets one coagulation factor, like thrombin or FXa. The efficacy for these new anticoagulants in APS patients remains to be established.

There are novel targets for drug development for treatment of patients with auto-antibodies against β2GPI. These drugs should interfere with binding of the antibodies to β2GPI, inhibit the binding of β2GPI to anionic surfaces or prevent binding of β2GPI-antibody complexes to receptors on cells. Ioannou et al. developed a recombinant peptide resembling domain I and showed in a mouse thrombosis model that these peptides can function as decoy and can neutralize the effects of anti-β2GPI antibodies. Injection of mice with these peptides lead to a smaller thrombus size after an induced vessel wall injury. A second idea based on the use of peptides is the blocking of the phospholipid binding site in β2GPI, thereby preventing its interaction with phospholipids leading to a diminished interaction of antibodies with β2GPI. A third inhibitor of pathogenic β2GPI is the dimeric A1-A1. A1 is the part of Apolipoprotein ER2 (ApoER2), a receptor expressed on platelets, endothelial cells and monocytes that interacts with domain V of β2GPI. This interaction results in activation of these cells and animal studies have shown that deficiency of this receptor attenuates the effects of antiphospholipid antibodies in a mouse model of thrombosis. Although these three approaches could all be promising it remains to be established whether this works in vivo. Furthermore, it should be excluded that the use of peptides mimicking parts of β2GPI trigger the formation of autoantibodies towards β2GPI.

FUTURE DIRECTIONS

To complete our findings of the etiology of the anti-β2GPI antibodies it needs to be investigated whether these antibodies have the same clinical characteristics as
observed for APS patients. The conformational switch of $\beta_2$GPI from a closed circular to an open fish hook conformation can be considered the common denominator of the occurrence of auto-antibodies against $\beta_2$GPI. However, many questions remain. The experiments with protein H (Chapter 3) showed that multiple protein boosts are needed before auto-antibodies directed against $\beta_2$GPI can arise. We need a better understanding under which conditions $\beta_2$GPI opens up and which time window is necessary for the induction of these auto-antibodies. This automatically leads to the second important question: what are the determinants that result in a switch from temporary to permanently presence of auto-antibodies towards $\beta_2$GPI. Current data are insufficient to answer this question. It is clear that antibodies directed against $\beta_2$GPI are not uncommon during infectious diseases and are transiently present. The conditions to maintain the persistent presence of anti-$\beta_2$GPI antibodies as observed for APS patients is unknown. By gaining more insight in these conditions, these can possibly be avoided and APS can be prevented.
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