Chapter 8  \hspace{1cm} \textbf{General discussion}
The association between the persistent presence of antiphospholipid antibodies (aPL) in plasma and the development of thrombotic complications has been termed the antiphospholipid syndrome (APS). Nowadays, it is known that these so-called 'antiphospholipid antibodies' are not directed against phospholipids directly, as is implicated by their name, but against phospholipid-bound proteins, of which $\beta_2$-glycoprotein I ($\beta_2$GPI) and prothrombin are most important ones. These antiphospholipid antibodies are routinely detected in two ways: (1) by their ability to bind to the negatively charged phospholipid cardiolipin using an enzyme-linked immunosorbent assay (ELISA) set-up (only for anti-$\beta_2$GPI antibodies) and (2) by their ability to prolong phospholipid-dependent coagulation assays, which is called lupus anticoagulant (LAC) activity. The most powerful way to measure pathogenic antibodies is to determine LAC activity, since it has been shown that the presence of LAC positive antibodies in plasma is not only an independent risk factor for the development of thrombosis in patients with the antiphospholipid syndrome, but also the risk factor with the strongest correlation $^{1-5}$. Furthermore, the LAC assay is a kind of a functional test for the detection of these antibodies and therefore LAC positive antibodies can be considered as ‘functional’ antibodies.

The discovery that antiphospholipid antibodies were not directed against phospholipids directly, but against phospholipid-bound proteins such as $\beta_2$GPI, has stimulated the research on the paradox between the development of thrombosis and LAC activity enormously. It is known now that antiphospholipid antibodies, especially anti-$\beta_2$GPI antibodies, in vitro have stimulating effects on endothelial cells, platelets, monocytes and influences the coagulation reactions, but a clear mechanism of action by which anti-$\beta_2$GPI antibodies can cause thrombosis has never been described.

In this thesis, using fusion proteins of $\beta_2$GPI and the dimerization domain of coagulation factor XI as a model for $\beta_2$GPI-anti-$\beta_2$GPI antibody complexes, an in vitro thrombosis model for the antiphospholipid syndrome was developed to investigate a mechanism by which antiphospholipid antibodies can cause thrombosis.

$\beta_2$GPI-anti-$\beta_2$GPI complexes

Two mechanisms have been described by which anti-$\beta_2$GPI antibodies can form a complex with $\beta_2$GPI and subsequently exert their in vitro effects. First, one anti-$\beta_2$GPI antibody binds two molecules $\beta_2$GPI, thereby forming a bivalent complex $^{6-8}$ (fig. 1A), and second one anti-$\beta_2$GPI antibody binds one $\beta_2$GPI molecule, which results in a conformational change in $\beta_2$GPI $^{9-15}$ (fig. 1B). Both these mechanisms result in an enormously increased affinity for negatively charged phospholipids compared to $\beta_2$GPI alone. Using our chimeric constructs of $\beta_2$GPI and the dimerization domain of coagulation factor XI, we have shown that the formation of a bivalent complex between $\beta_2$GPI and anti-$\beta_2$GPI antibodies is enough to mimic the effects of $\beta_2$GPI-anti-$\beta_2$GPI antibody complexes with respect to binding to phospholipids and LAC activity (chapter 3). Although it is also shown that the F(ab) fragment of a LAC positive anti-$\beta_2$GPI antibody complexed to $\beta_2$GPI is not able to induce LAC activity, one cannot exclude the possibility that the second mechanism does not play a role at all. A recent publication of Hammel et al. suggests that $\beta_2$GPI in solution has a slightly different
conformation compared to β2GPI bound to a membrane. The change in conformation is found in domain II, a domain that is not involved in binding to phospholipids. We cannot exclude completely that the fusion of the dimerization domain of factor XI to the N-terminus of β2GPI induces a conformational change in the domain V, which harbours the site that is essential for binding to phospholipids of β2GPI. However, this is unlikely, because fusion of β2GPI with a comparable domain, apple2 of factor XI, did not induce such a conformational change.

**FIG. 1.** Binding of β2GPI-anti-β2GPI antibody complexes to phospholipid membranes. This can occur by the formation of a bivalent complex between β2GPI and an anti-β2GPI antibody (A) or by the induction of a conformational change in β2GPI after binding to an anti-β2GPI antibody (B). Both result in a highly increased affinity for phospholipids compared to β2GPI alone.
A mechanism for the development of thrombosis

It has been shown that antiphospholipid antibodies, in particular anti-β₂GPI antibodies, are able to activate cells such as platelets, endothelial cells and monocytes 17-24. Negatively charged phospholipids exposed on the membranes of these cells can interact with β₂GPI-anti-β₂GPI antibody complexes, which then results in an increased expression of activation markers. It has been proposed that Fc-receptors play a role in this activation 25. However, recently it has been shown that F(ab)₂ fragments of an anti-β₂GPI antibody and the complete anti-β₂GPI antibody are equally able to induce increased thrombus formation in a hamster thrombosis model 26. This implicates that, since binding of a protein to phospholipids alone is not enough to activate cells, as was obvious from the inability of annexin V to activate platelets 27, protein receptors must play a role. We showed that both dimers of β₂GPI and anti-β₂GPI antibodies stimulate platelet adhesion to collagen in an in vitro flow model. Further, it was shown that this increase in adhesion was mediated by binding of dimeric β₂GPI or β₂GPI-anti-β₂GPI antibody complexes to a receptor of the low-density lipoprotein (LDL) receptor family, because the increased adhesion could be inhibited by receptor-associated protein (RAP)(chapter 4). We propose a model by which β₂GPI-anti-β₂GPI antibody complexes can cause (arterial) thrombosis. As shown in fig. 2, β₂GPI-anti-β₂GPI antibody complexes bind to negatively charged phospholipids that are exposed on the membrane of platelets after their activation. This is followed by binding of the β₂GPI-anti-β₂GPI antibody complex to a receptor of the LDL-receptor family on the platelet membrane and subsequently dimerization of this receptor. This results in the transduction of signals by which the platelets become sensitized and are more prone to form a thrombus after exposure to another stimulus. In chapter 7, we described that dimeric β₂GPI is able to bind to the LDL receptor-related protein (LRP) and megalin. This makes it more likely that LRP8Δ4-6, which is the only member of the LDL-receptor family present on platelets, is the receptor by which β₂GPI-anti-β₂GPI antibody complexes exert their pathogenic effects. Furthermore, we showed that binding of dimeric β₂GPI to LRP is inhibited by anti-β₂GPI antibody 4F3, an antibody that also inhibited the increased platelet deposition on collagen in the in vitro flow system. This suggests that this antibody interferes with the binding of dimeric β₂GPI to its receptor, thereby inhibiting the effects of the dimer. However, it is not excluded that under certain conditions the Fc-receptor plays an additional role.

It is quite obvious that the mechanism mentioned above may also fit for the activation of other cell types, such as endothelial cells (via the very-low-density lipoprotein receptor), monocytes and cells of the placenta (via LRP) and the kidney (via megalin), but these receptors may also play a role in normal physiology by binding β₂GPI. For example, megalin can bind β₂GPI alone at a concentration of 0.2 µM, which is 20 times lower than the plasma concentration of β₂GPI. It has been suggested that megalin has a role in the clearance of phosphatidylserine-containing particles 28. Recently, it was reported that β₂GPI inhibits both low-density lipoprotein oxidation and cholesterol accumulation by macrophages in vitro 29. This inhibition of cholesterol accumulation was a result of a reduction of cholesterol influx and an increase in cholesterol efflux. Since the uptake of cholesterol is mediated by members of the LDL-receptor family, an explanation for the inhibition of cholesterol accumulation may be that β₂GPI interacts with these receptors, thereby preventing the uptake of cholesterol. These observations all
are of interest by elucidating an (up to now) unknown physiological function of β₂GPI.

**FIG. 2.** Model for the mechanism by which β₂GPI-anti-β₂GPI antibody complexes can cause thrombosis. The β₂GPI-anti-β₂GPI antibody complexes bind to the negatively charged phospholipids exposed on the platelet membrane (A). This is followed by the binding of these complexes to a receptor of the LDL-receptor family (B). Subsequently, this receptor dimerizes and signalling starts. Hereby the platelets become sensitized and are more prone to form thrombi (C).
Anti-prothrombin antibodies

At present, there is no consensus on the prothrombotic potential of anti-prothrombin antibodies. Conflicting results about the association between thrombosis and the presence of anti-prothrombin antibodies have been described.

We showed in the in vitro flow model that anti-prothrombin antibodies do not influence platelet deposition to collagen at an arterial flow rate (chapter 5). A likely explanation for this observation was the observation that anti-prothrombin antibodies do not bind to platelets adhered to collagen. This is remarkable, because platelets adhered to collagen do expose negatively charged phospholipids, by which they can interact with prothrombin-anti-prothrombin antibody complexes. As it is clearly different from what we observed for β2GPI-anti-β2GPI antibody complexes, this may indicate that sensitisation of platelets does not only involve interaction with negatively charged phospholipids, but that this interactions needs to be stabilized by the binding of the antigen-antibody complex to a receptor. In this respect, it is interesting to note that the amount of antiphospholipid antibodies in serum is comparable to the amount in plasma, despite extensive activation of platelets during serum formation. These results do not exclude that anti-prothrombin antibodies are pathogenic. It is possible that the low molecular weight heparin used as anticoagulant in these experiments interferes with binding of the prothrombin-anti-prothrombin antibody complex to a receptor, although this is difficult to circumvent: the binding of prothrombin to negatively charged phospholipids is dependent on the presence of calcium ions, which implies that citrate and EDTA cannot be used as anticoagulants. On platelets, one receptor for prothrombin has been described: αmβ3. The affinity of prothrombin for this receptor is low compared to the affinity of fibrinogen and von Willebrand factor (vWF) for αmβ3. However, the affinity of prothrombin for this receptor may increase by the formation of a bivalent complex between prothrombin and anti-prothrombin antibodies. If this mechanism is involved, the prothrombotic effects of prothrombin-anti-prothrombin antibody complexes are not detected in a perfusion experiment using collagen as a surface, because platelet adhesion to collagen is relatively independent on αmβ3. αmβ3 is necessary for the formation of aggregates on collagen, but as no effect on aggregate formation has been detected prothrombin-anti-prothrombin antibody complexes apparently did not influence the formation of a bridge between two αmβ3 molecules on different platelets.

The balance between thrombosis and bleeding complications

The mechanism by which antiphospholipid antibodies cause thrombosis is not clear at present. Several mechanisms by which these antibodies can cause thrombosis have been reported. In general, these can be divided into three groups: (1) interference with the coagulation cascade, (2) activation of the complement system, and (3) activation of cells involved in haemostasis. The latter has been discussed in the section ‘a mechanism for the development of thrombosis’ in this chapter. The interference with the coagulation cascade will be discussed now. During haemostasis, very low levels of thrombin, which are able to activate protein C to activated protein C (APC), are present in the circulation 31,32. Therefore,
small amounts of APC continuously inhibit coagulation, preventing further activation of the coagulation cascade. In *in vitro* coagulation assays (used for the detection of LAC activity) this protein C pathway does not play a role, because via the high concentrations of activators used in *in vitro* coagulation assays, high concentrations of thrombin are formed. This phenomenon, in which low concentrations of thrombin have anti-thrombotic and high levels of thrombin have prothrombotic effects, is named the thrombin paradox. It can be speculated that *in vivo* antiphospholipid antibodies may interfere with the formation of low levels of thrombin, thereby inhibiting the formation of low levels of APC. On the other hand, *in vitro* antiphospholipid antibodies in plasma compete with the clotting factors for binding to available phospholipid surfaces, and thereby prolong clotting times. This suggests that *in vivo* the presence of antiphospholipid antibodies can result in a prothrombotic state, whereas *in vitro* the presence of these antibodies results in prolongation of coagulation. Our group previously reported that the presence of antiphospholipid antibodies in patient plasma have no effect on circulating APC levels *in vivo*. However, it also has been noted that infusion of APC in mice immunized with antiphospholipid antibodies significantly decreases thrombus size after injury.

Activation of the complement system may also play a role in the mechanism by which these antibodies exert their prothrombotic effects. Holers *et al.* have shown that activation of the complement system is important for antibody-induced fetal loss and growth retardation, but did result in normal litter sizes in a mice model. However, as the anticardiolipin antibodies used in this model did not recognize murine β₂GPI-cardiolipin complexes, thus the results described are not easy to understand.

Thus, at present three possible mechanisms for the development of thrombosis in patients with anti-β₂GPI antibodies have been proposed: (1) interference of antiphospholipid antibodies with the protein C pathway, (2) activation of the complement system, and (3) activation of cells involved in haemostasis. The latter can be subdivided into (a) activation via a Fc-receptor-dependent mechanism, and (b) activation of these cells via a receptor specific for β₂GPI-anti-β₂GPI antibody complexes. These mechanisms are all based on *in vitro* observations and observations in animal models. However, the value of most of the observations in animal models for the antiphospholipid syndrome is limited. Most of these animal models are based on the induction of experimental ‘antiphospholipid syndrome’ with antibodies directed against human β₂GPI and the majority of the studies did not test if these antibodies recognize murine β₂GPI. In the absence of such reactivity, the artificial experimental ‘antiphospholipid syndrome’ differs from the human situation. In these experimental ‘antiphospholipid syndromes’, activation of Fc-receptors via aggregated IgG and the complement system then would be the major explanation and not specific activation of platelets and endothelial cells by β₂GPI-anti-β₂GPI antibody complexes. In a hamster thrombosis model, in which it was shown that the anti-β₂GPI antibodies used recognize hamster β₂GPI, the increase in thrombus size was not dependent on activation of the Fc-receptor. This clearly supports a role for a receptor that is specific for β₂GPI-anti-β₂GPI antibody complexes. Our experiments described in chapter 4 confirm these observations. However, these observations are only relevant for arterial thrombosis in which the activation of platelets is important. For venous thrombosis, the formation of an extensive fibrin network is the dominant factor. This suggests that activation of the coagulation cascade (or inhibition of the protein C pathway)
is an important mechanism by which antiphospholipid antibodies cause venous thrombosis. However, this has never been studied in \textit{in vivo} animal models. It is possible that all mechanisms play a role \textit{in vivo} under different conditions. Furthermore, binding of the $\beta_2\text{GPI}$-anti-$\beta_2\text{GPI}$ antibody complexes to negatively charged phospholipids, which is, as was shown in chapter 4, essential for activation of platelets, may \textit{in vivo} also result in inhibition of coagulation. But the balance between the activation of cells, such as platelets and endothelial cells, and the interference with the protein C pathway (thrombotic) and the inhibition of coagulation (anti-thrombotic) will finally result in the development of a pathological thrombus in patients with the antiphospholipid syndrome.

\textbf{Future prospectives: what about the patient?}

In the future, the search for the mechanism by which these antibodies cause thrombosis will be continued by us and other groups. An important question is: do these antibodies activate platelets, endothelial cells and monocytes via similar mechanisms? However, the most important question will be: are the mechanisms by which $\beta_2\text{GPI}$-anti-$\beta_2\text{GPI}$ antibody complexes stimulate the development of thrombosis \textit{in vivo} similar to the mechanisms concluded from \textit{in vitro} experiments?

Another important problem is the treatment of patients with the antiphospholipid syndrome. Currently, most patients with the antiphospholipid syndrome are treated for life with oral anticoagulants and aspirin to prevent a recurrent thrombosis. However, such treatment needs careful and regular measurement of the level of anticoagulation and there is always a possibility for bleeding complications. Heparin, is a well-known and effective therapeutic anticoagulant \textsuperscript{36}. \textit{In vitro} experiments suggest that heparin inhibits some specific effects of antiphospholipid antibodies, in particular related to pregnancy problems. It has been shown that heparin restores trophoblast function and invasiveness, which are disturbed in the presence of antiphospholipid antibodies, in an \textit{in vitro} model. It has also been suggested that heparin may act as an immunomodulatory agent, which affects cytokine production and cell signalling \textsuperscript{37}. Two possible mechanisms by which heparin inhibits the effects of $\beta_2\text{GPI}$-anti-$\beta_2\text{GPI}$ antibody complexes can be suggested. First, heparin inhibits the binding of these complexes to negatively charged phospholipids by binding to the phospholipid binding site of $\beta_2\text{GPI}$. Second, heparin may inhibit the binding of $\beta_2\text{GPI}$-anti-$\beta_2\text{GPI}$ antibody complexes to a receptor of the LDL-receptor family. Both these mechanisms will result in abolishment of the effects of $\beta_2\text{GPI}$-anti-$\beta_2\text{GPI}$ antibody complexes. We have shown in chapter 7 that heparin indeed inhibits the binding of dimeric $\beta_2\text{GPI}$ to LRP. These findings suggest that, compared to oral anticoagulants, treatment with heparin may be preferable. As a long term treatment with heparin is not possible, studies in which the influence of heparin and low molecular weight heparin (LWMH) on phospholipid binding and interaction with LRP are compared, should be performed. Bleeding complications with prophylactic doses of LMWH are also less frequent compared to oral anticoagulant use. However, induction of osteoporosis with long term use argue against its use.

It would be very interesting to determine the effects of antibody 4F3 on the activation of other cell types by dimeric $\beta_2\text{GPI}$. When this antibody also inhibits the activation of other
cells, like endothelial cells and monocytes, it might be a possible way to inhibit the activation of cells by $\beta_2$GPI-anti-$\beta_2$GPI antibody complexes and thereby prevent the development of thrombosis in patients with the antiphospholipid syndrome. Theoretically, infusion of a F(ab)$_2$ fragment of this antibody would be a new therapy to prevent recurrent thrombosis. However, one of the most important problems will be the clearance of the $\beta_2$GPI-F(ab)$_2$ complex and thus a very short half life of the protein, which makes it with the current knowledge not useful.

Another novel approach to develop a therapy for patients with the antiphospholipid syndrome, is to induce tolerance against $\beta_2$GPI in these patients. Recently, it has been reported that the majority of the anti-$\beta_2$GPI antibodies in patients with the antiphospholipid syndrome are directed against domain I of $\beta_2$GPI 38,39. This knowledge was used to make a polyvalent construct that might induce tolerance of anti-$\beta_2$GPI secreting B-cells. To do so, four molecules of domain I of $\beta_2$GPI were coupled to one molecule of polyethyleneglycol (PEG, 20 kDa; LPJ 1082). These molecules are used to induce polyclonal activation of premature B cells, which will then result in apoptosis of these B cells. This system seems to work in mice, where administration of domain I of $\beta_2$GPI gave inhibition of the antibody response to $\beta_2$GPI with a decrease in number of antibody forming cells. At present, this therapy is tested in a phase I and II clinical trial 40. However, some critical remarks have to be made. Can these molecules discriminate between premature and mature B cells? And do these molecules really induce tolerance against anti-$\beta_2$GPI antibodies or is inhibition of the antibody response just a decrease in circulating anti-$\beta_2$GPI antibodies caused by the clearance of complexes between anti-$\beta_2$GPI antibodies and LPJ 1082? Furthermore, an important difference between the model in mice and humans is that treatment in mice starts much earlier than in humans. In humans one will treat an ongoing disease, which is much more difficult to treat than a disease that had just started in most cases.

Thus, in the future there might be better therapies for patients with the antiphospholipid syndrome. However, it will be difficult to develop one standard treatment for these patients, because the population of autoantibodies involved in this syndrome seems to be very heterogeneous. Therefore, the effects of $\beta_2$GPI-anti-$\beta_2$GPI antibody complexes on various cell types, such as platelets, endothelial cells and monocytes, will be subject of ongoing research. Furthermore, the effects of prothrombin-anti-prothrombin antibody complexes on these cells need to be investigated in more detail to get more insight in the mechanism by which these antibodies can cause thrombosis.
References


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