

Pathophysiology of antiphospholipid antibodies

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ABSTRACT

The presence of antiphospholipid antibodies in plasma is a risk factor for thromboembolic complications. *In vitro*, however, the same antibodies can prolong clotting times in coagulation assays, a classic marker for a bleeding tendency. For years this contradiction has puzzled many scientists. Recently new insights into the interaction between antiphospholipid antibodies and their main target, the protein beta-2-glycoprotein I, have opened new avenues for the understanding of the pathology of this syndrome.

The only two antibodies that are frequently present are anti- β_2 -GPI antibodies and antiprothrombin antibodies; of these the most important and relevant protein involved in APS seems to be β_2 -GPI. Beta-2-GPI is a plasma protein with no obvious function and persons or mice lacking this protein seem to be completely healthy.⁷

The antiphospholipid syndrome is a very unusual syndrome because the clinical symptoms such as thrombosis occur relatively often but in most cases are not due to the

INTRODUCTION

The antiphospholipid syndrome (APS) is a noninflammatory autoimmune disease characterised by the presence of antiphospholipid antibodies (aPL) in the plasma of patients with venous and/or arterial thrombosis and/or recurrent complications of pregnancy.^{1,2} The presence of aPL in plasma of patients can be detected by either a prolongation of the phospholipid dependent coagulation test (lupus anticoagulant, LAC), or by solid phase immune assays (anticardiolipin ELISA).³ Antiphospholipid antibodies that cause LAC activity and anticardiolipin antibodies (aCL) are closely related but not identical autoantibodies. Originally, it was thought that aPL were directed against anionic phospholipids. We now know that the antibodies are directed against plasma proteins with affinity for anionic phospholipids. After the discovery of β_2 -glycoprotein I (β_2 -GPI) as an important antigen in the anticardiolipin ELISA,^{4,6} over time a large number of possible other target proteins have been described (table 1).

Table 1

List of published targets for antiphospholipid antibodies. The assays used in diagnostic laboratories to detect the presence of these antibodies recognise β_2 -glycoprotein I (LAC assay and anticardiolipin ELISA) or prothrombin (LAC assay). The presence of antibodies to the other targets is not detected in a normal diagnostic setting. The relevance of all other antibodies is questionable.

LIPID / POLYSACCHARIDE SUBSTRATES	PROTEIN SUBSTRATES
Cardiolipin	β_2 -glycoprotein I
Phosphatidylserine	Prothrombin
Phosphatidyl ethanolamine	Protein C
Phosphatidyl inositol	Protein S
Phosphatidic acid	High-molecular weight kininogen
Lysobisphosphatidic acid	Factor XII
Heparan sulphate	Annexin A5
Oxidised low-density lipoprotein	Tissue factor pathway inhibitor
7-ketocholesteryl-9-carboxy-nonoate	Complement factor H
	Phospholipases
	Plasminogen
	Tissue-type plasminogen activator

presence of antiphospholipid antibodies. The detection of the antibodies in the blood of a patient with thrombosis or complications of pregnancy is an essential step to define the syndrome. However, a major assay to detect the antibodies is a prolongation of a coagulation assay and normally prolongation of a clotting test is used to detect a bleeding disorder and not a risk for thrombosis. Given this contrast between the *in vivo* clinical manifestations and the laboratory observations, a large number of hypotheses to explain the pathology of the syndrome have been proposed.^{8,9} The first suggested mechanisms were based on the interference of antiphospholipid antibodies with protein-protein or protein-phospholipid interactions that are essential for optimal haemostasis. However, none of the proposed mechanisms survived studies in which the hypothesis was tested with plasma samples of larger cohorts of patients. Nowadays, possible stimulation of blood and endothelial cells by antiphospholipid antibodies has been emphasised. Two important assumptions must be considered to understand a possible cellular action of antiphospholipid antibodies. Firstly, the pathological autoantibodies are not directed against phospholipids *per se*, but against a protein, β_2 -GPI. Secondly, it is now generally accepted that aPL do not inhibit the functional activity of β_2 -GPI but they do induce a new function for β_2 -GPI, namely a significantly increased affinity for cellular surfaces containing anionic phospholipids. Thus, the affinity of β_2 -GPI for negatively charged phospholipids only becomes high enough to interact with cells after interaction with the antibodies. In this overview, we will discuss current insights into the protein β_2 -GPI, how it interacts with antibodies and (cellular) surfaces and the consequences of the binding of protein-antibody complexes to the cell on cellular functions.

BETA-2-GLYCOPROTEIN I

Beta-2-glycoprotein I is a glycoprotein present in plasma at concentrations ranging from 10 to 300 $\mu\text{g/ml}$ (0.25-5.0 μM).¹⁰⁻¹² Messenger RNA is found in endothelial cells,¹³ placenta,¹⁴ central nervous system cells¹⁵ and hepatocytes,¹⁶ but its major source of synthesis is the liver.¹⁷ Beta-2-GPI is synthesised as a 326 amino acid long single chain polypeptide with a calculated molecular mass of 36.3 kDa.¹⁷ It contains four potential glycation sites and the glycans account for approximately 20% (w/w) of the total molecular mass of about 45kD as determined by SDS-PAGE gelelectrophoresis.¹⁷ As early as in 1968, a deficiency of β_2 -GPI was described without any clinical consequences,¹⁸ an observation that has since been confirmed several times.^{10,11,19}

The mature sequence of β_2 -GPI consists of five repeating units of the same type, termed short consensus repeat

(SCR) domains.¹⁷ SCR domains are present in many proteins functioning in the complement system.²⁰ They consist of about 60 residues and they have two fully conserved disulphide bonds. Sequence homology among SCR domains ranges between 20 and 40%. Beta-2-glycoprotein I is built up out of four regular SCR domains and one aberrant domain. This fifth domain contains a six-residue insertion and a 19-residue C-terminal extension, which is C-terminally cross-linked by an additional C-terminal disulphide bond.

The crystal structure of β_2 -GPI reveals an extended chain of SCR domains in a fishhook-like form (figure 1).^{21,22} The first four domains have common SCR forms. The fifth domain deviates strongly from the common SCR folding. Similar to the other domains it has the central antiparallel β -sheets and the common two disulphide bonds.

However, half of the domain, in particular the parts that contain the 6 amino acid insertion and the 19 amino acid

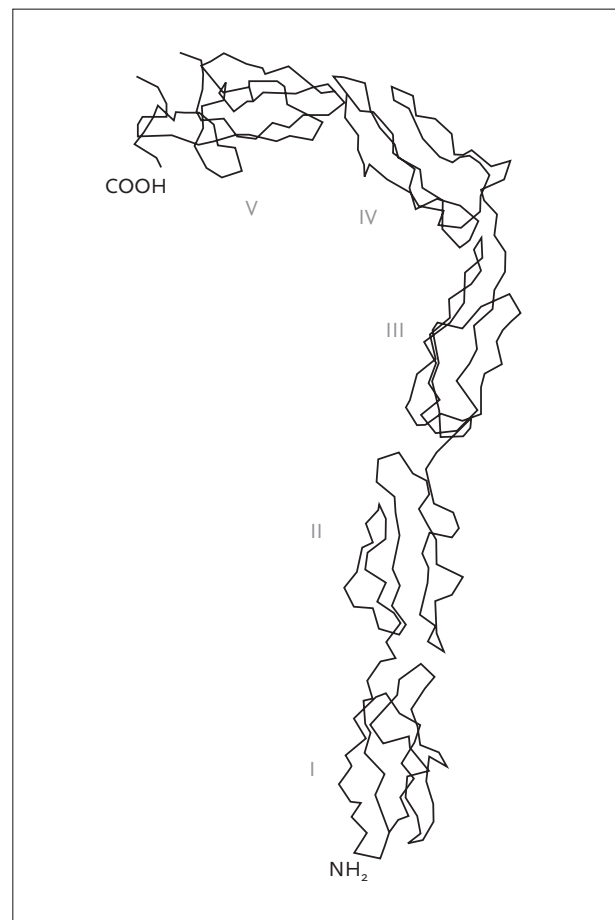


Figure 1

The three-dimensional structure of β_2 -glycoprotein I. β_2 -glycoprotein I consists of five comparable SRC repeats. The phospholipid binding site is located in domain V. This domain contains a flexible loop that is not visible in the crystal structure. This loop penetrates into the membrane when β_2 -glycoprotein I binds to cellular membranes.

extension, form a unique structural element which is the phospholipid binding site. A 2000Å large patch of 14 positively charged amino acids provides the electrostatic interactions with the anionic head groups of the phospholipids. In the middle of this patch a hydrophobic group is present that can insert into the membrane, thereby anchoring the protein to the membrane. Amino acid replacement studies have shown that the presence of hydrophobic amino acids in this loop is crucial for phospholipid binding.²³

The binding site for phospholipids is located in the fifth domain at the outer curve of the fishhook, the top of the molecule. When β_2 -GPI binds to a membrane surface, the domains I and II are exposed far away from the cellular surface. As domains III and IV are heavily glycosylated and therefore shielded from protein-protein interactions, domains I and II are ideally exposed for interactions with other proteins and can provide binding sites for anti- β_2 -GPI antibodies, for example.

SPECIFICITY OF ANTIBODIES

Antiphospholipid antibodies is a generic term that describes a collection of closely related but not identical antibodies: LAC activity, anticardiolipin antibodies and anti- β_2 -GPI antibodies. This immediately raises two fundamental questions: what are the differences between the different types of antibodies and which one is the most relevant? A meta-analysis on the predictive value of the different types of aPL antibodies showed that the antibodies that induce LAC activity correlate best with a history of thromboembolic complications.^{24,25} Apparently, an assay that measures a functional activity, inhibition of a clotting reaction, better predicts a thrombotic risk than assays that measure the presence of autoantibodies that comprise both those that influence a functional activity and those that do not. But besides the fact that one assay is based on functional activity and the other not, there are more reasons why the assays do not measure an identical population of antibodies. In the first place LAC can also be caused by antiprothrombin antibodies.

Antiprothrombin antibodies are not detected in an anticardiolipin ELISA.²⁶ Antiprothrombin antibodies are probably of little clinical significance.²⁷ Secondly, the ELISAs developed to detect the presence of anticardiolipin or anti- β_2 -GPI antibodies are poorly standardised. A major reason for the poor comparison between the different types of antibodies might be that a plasma sample that is positive in one laboratory can be negative in another.^{28,29} Even between laboratories with extensive experience in the detection of aPL antibodies, discordant findings with low titre antibodies samples are more the rule than an exception.

Since 1990, it is known that the pathological anticardiolipin antibodies are in fact anti- β_2 -GPI antibodies. To better understand the pathophysiology of anti- β_2 -GPI antibodies it is important to characterise the epitopes on β_2 -GPI involved in the recognition by the autoantibodies. The first published experiments suggested that anti- β_2 -GPI antibodies were a heterogeneous group of antibodies because antibodies were found directed against every possible epitope on the protein.³⁰⁻³⁴ However, the assays to detect the presence of autoantibodies are rather aspecific and proper standardisation of the anti- β_2 -GPI-antibody ELISA is lacking.^{28,29} Improvements in the detection of the antibodies by preventing the binding of low affinity, aspecific antibodies and the use of deletion mutants of β_2 -GPI have resulted in strong evidence that the major, if not the only, epitope on β_2 -GPI responsible for the binding of pathological autoantibodies is situated in domain I, probably near Lysine 43.^{35,36}

In summary, there is a heterogeneous population of antiphospholipid antibodies but only a subpopulation of these antibodies is pathological. One of the major challenges is to improve our serology in such a way that specifically the pathological antibodies are detected.

CONSEQUENCES OF ANTIBODY BINDING

Originally, it was thought that aPL antibodies prolonged clotting times by means of competition with clotting factors for binding to negatively charged phospholipids that are essential for optimal coagulation. The discovery that not negatively charged phospholipids but β_2 -GPI was the antigen pointed to another explanation. Beta-2-GPI on its own has a relatively low affinity for negatively charged phospholipids. However, the presence of anti- β_2 -GPI antibodies causes two phospholipid-bound β_2 -GPI molecules to cross-link, thereby increasing its affinity a hundred-fold.³⁷⁻³⁹ Only the antibody- β_2 -GPI complexes are able to interfere with the binding of clotting factors with their catalytic phospholipid surface, not β_2 -GPI alone (*figure 1*). Studies in a hamster model with monoclonal anti- β_2 -GPI antibodies showed that the antibodies and not their Fab fragments induce increased thrombus formation, indicating that the dimerisation of β_2 -GPI by antibodies is essential not only for the induction of LAC but also for the induction of thrombotic complications.⁴⁰ Recently, we showed that dimerisation is also essential for the activation of platelets by β_2 -GPI.⁴¹ All these studies indicate that the anti- β_2 -GPI antibodies are gain-of-function antibodies. They induce a new function in β_2 -GPI, namely an increased affinity for negatively charged phospholipids. Beta-2-GPI on its own is unable to interfere with membrane-bound reactions, it

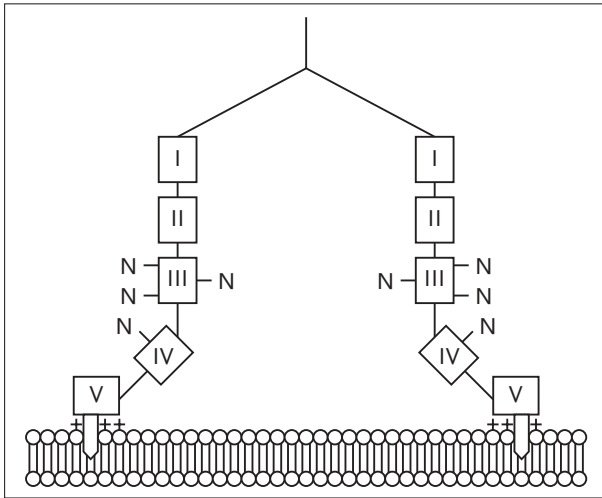


Figure 2

Mechanism by which anti- β_2 -glycoprotein I antibodies increase the affinity of β_2 -glycoprotein I for phospholipids. The antibodies dimerise β_2 -glycoprotein I thereby doubling the number of binding sites. Without antibody, the affinity for a PS/PC surface is 170 nM, with antibody the affinity increases to 5nM. The affinity of the complex is now high enough to compete with clotting factors for the available catalytic phospholipids in clotting assays. (N = carbohydrate side chain).

can only interfere with physiological functions after an interaction with antibodies. These observations are now the lead in the hypotheses that explain the pathophysiology of the antibodies.

PATHOPHYSIOLOGY

Antiphospholipid antibodies are notable because they increase the risk for both venous and arterial thrombosis. Almost all other known risk factors increase only venous or only arterial thrombosis.⁴² In general, markers related to humoral coagulation factors result in venous thrombosis while markers related to platelets correlate with arterial thrombosis. We cannot exclude that the risk for arterial thrombosis and the risk of venous thrombosis are the result of two separate actions of β_2 -GPI-antibody complexes. The most important mechanisms that have been put forward by which anti- β_2 -GPI could increase a thrombotic risk is that they interfere with phospholipid dependent antithrombotic pathways or that they bind to blood and/or endothelial cells, thereby activating these cells. The major antithrombotic pathway is the protein C pathway. Protein C, activated by thrombin bound to thrombomodulin, cleaves factors Va and VIIIa thereby preventing further thrombin formation.⁴³ The whole protein C reaction

cascade takes place on a phospholipid surface. Indeed, 'protein C resistance' has been found in patients with antiphospholipid antibodies *in vitro*.⁴⁴ Whether this is also very relevant *in vivo* is unknown. Apparently the antibodies inhibit both the prothrombotic pathway (coagulation) and the anticoagulant pathway (protein C axis) and the overall result may be neutral. Some authors have reported that the presence of phosphatidyl ethanolamine in lipid vesicles might shift the balance towards a more pronounced inhibition of the antithrombotic pathway.⁴⁵ More information (animal experiments) is necessary to judge a possible role of acquired protein C resistance as an important pathological mechanism in the antiphospholipid syndrome. Recently, evidence has revealed that β_2 -GPI-antibody complexes are able to activate platelets, monocytes and endothelial cells.⁴⁶⁻⁴⁸ Activation of these cells results in increased platelet activation and induction of tissue factor activity, the major inducer of the coagulation cascade. The activation of the cells is not due to binding to phospholipids on the surface of the cells but to binding to specific receptors on the cells. A number of receptors have been suggested, apoER2' on platelets, annexin A2 on monocytes and a Toll-like receptor on endothelial cells. It is remarkable that β_2 -GPI only has affinity for these receptors when bound to an antibody. This can be explained in two ways. The binding of β_2 -GPI-antibody to the phospholipids of the membrane is an essential condition before β_2 -GPI can bind to a receptor. Thermodynamically, this can be understood because binding to a membrane reduces the entropy of the reaction, also allowing low-affinity interactions. A second explanation is that binding of the antibodies to β_2 -GPI induces a conformational change into β_2 -GPI, exposing a neo-epitope that is involved in the interaction with the receptor.⁴⁹ The activation of cells by antiphospholipid antibodies is normally weak and not enough to fully activate the cell. It is now generally believed that antiphospholipid antibodies make the cells more sensitive for other activators or that other activators, in the presence of the antibodies, can activate cells at a lower concentration. A second hit is necessary. This explains why although the antibodies are permanently present in the plasma of patients, the patients do not suffer continuously from thrombotic complications. Only the risk to develop thrombosis or pregnancy complications is increased.

CONCLUSIONS

We have now reached a fascinating area in the research into the pathology of the antiphospholipid syndrome. New findings on the specificity of the antibodies open the possibility to develop better and more specific assays for the detection of patients at risk for thromboembolic

complications. With a better definition of the patients that really suffer from the syndrome, future patient studies will not be disturbed by the inclusion of incorrectly classified patients within the patient cohorts. The notion that anti-phospholipid antibodies do not inhibit a certain metabolic function but that due to binding of the antibodies to β_2 -GPI a new function for β_2 -GPI is induced was a major step forward. The research into the pathology of the syndrome was also blinded too much by the idea that negatively charged phospholipids were the central theme in the explanation of the syndrome. Nowadays we envision the concept of cells and cell activation as the consequence of 'classic' receptor-substrate interactions as the major cause of the pathophysiology. With the new tools and ideas that have been developed in the last few years we are now able to test the current hypotheses in animal models and possibly large patient cohort studies. It is, of course, still unknown whether the pathology of venous thrombosis is the same as the pathology of arterial thrombosis.

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