β_2-glycoprotein I

Its role in the antiphospholipid syndrome

Bianca C. H. Lutters

Cover illustration: J. Ruitenburg

Printed by: Optima, Rotterdam

ISBN: 90-393-3250-9

β_2-glycoprotein I

Its role in the antiphospholipid syndrome

β_2-glycoprotein I
De rol in het antifosfolipiden syndroom
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit van Utrecht op gezag van de Rector Magnificus,
Prof. Dr. WH Gispen, in gevolge het besluit van het
College voor Promoties in het openbaar te verdedigen
op dinsdag 4 februari 2003 des middags te 14.30 uur

door

Bianca Catharina Henrica Lutters

geboren op 18 januari 1976, te Weert

Promotoren: Prof. Dr. J.W.J. Bijlsma
Faculteit Geneeskunde, Universiteit Utrecht

Prof. Dr. Ph. G. de Groot
Faculteit Geneeskunde, Universiteit Utrecht

Co-promotor: Dr. R.H.W.M. Derksen
Faculteit Geneeskunde, Universiteit Utrecht

The study described in this thesis was supported by a grant of the Netherlands Heart Foundation (NHF-98.060).

Financial support by the Netherlands Heart Foundation for the publication of this thesis was gratefully acknowledged.

Additional financial support for the publication of this thesis by Dr. Ir. van de Laar Stichting, Sanofi-Synthelabo B.V., Pharmacia B.V., and NODIA B.V. is gratefully acknowledged.

Aan Rutger
Aan mijn ouders

Contents

Abbreviations

β_2GPI	β_2-glycoprotein I
aCL	anticardiolipin antibodies
ADP	adenosine 5'-diphosphate
ANOVA	analysis of variance
APC	activated protein C
aPL	antiphosphospholipid antibodies
ApoER2	apolipoprotein E receptor-2
APS	antiphospholipid syndrome
aPTT	activated partial thromboplastin time
AT	anti-thrombin
BCA	bichinchoninic acid
BSA	bovine serum albumine
C4BP	C4b-binding protein
CCP	complement control protein
CI-M6PR	cation-independent mannose-6-phosphate receptor
dPT	dilute prothrombin time
dRVVT	dilute Russell's viper venom time
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EPCR	endothelial cell protein C receptor
ER	endoplasmic reticulum
FCS	fetal calf serum
FII	prothrombin
FITC	fluorescein isothiocyanate
Gla	γ-carboxyglutamic acid
GPIb	glycoprotein Ib
GPVI	glycoprotein VI
HMWK	high molecular weight kininogen
HUVEC	human umbilical vein endothelial cell
KCT	kaolin clotting time
LAC	lupus anticoagulant
LDL	low-density lipoprotein
LMWH	low molecular weight heparin
LRP	LDL receptor-related protein
NO	nitric oxide
OPD	o-phenylene diamine
PAI	plasminogen activator inhibitor
PAPS	primary antiphospholipid syndrome
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PoAb	polyconal antibody
PPD	para-phenylene diamine
PRP	platelet-rich plasma
PS	phosphatidyl serine
PT	prothrombin time
RAMPO	peroxidase-labeled rabbit-anti-mouse antibody
RAP	receptor-associated protein
SAPS	secondary antiphospholipid syndrome
SCR	short consensus repeat
SD	standard deviation
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SLE	systemic lupus erythematosus
SPR	surface plasmon resonance
TAFI	thrombin activatable fibrinolysis inhibitor
TBS	tris-buffered saline
TF	tissue factor
TFPI	tissue factor pathway inhibitor
tPA	tissue-type plasminogen activator
TRITC	tetramethyl rhodamine isothiocyanate
TxA_2	thromboxane A_2
TxB_2	thromboxane B_2
uPA	urinary-type plasminogen activator
VLDL	very-low-density lipoprotein
vWF	von Willebrand factor

Chapter 1 | General introduction

The antiphospholipid syndrome

The term 'antiphospholipid syndrome' (APS) refers to patients with arterial or venous thrombosis, pregnancy loss, and thrombocytopenia, in which so-called antiphospholipid antibodies (aPL) are repeatedly detected in their plasmas. The antiphospholipid syndrome was originally described in patients with systemic lupus erythematosus (SLE), a systemic autoimmune disorder. The association between SLE with APS is called secondary antiphospholipid syndrome (SAPS). Over the years, APS in the absence of an underlying autoimmune disease has been recognized in many more patients (defined as primary antiphospholipid syndrome (PAPS)).

The antiphospholipid antibodies can be detected in an enzyme-linked immunosorbent assay (ELISA) and in *in vitro* coagulation assays. Traditionally, antibodies against the anionic phospholipid cardiolipin (aCL) are detected by ELISA. Using *in vitro* coagulation assays, antibodies are detected by their ability to prolong phospholipid-dependent clotting assays. These antibodies are termed lupus anticoagulants (LAC).

Historical background

The history of antiphospholipid antibodies started about 50 years ago with the observation that some SLE patients showed a biologically false-positive tests for syphilis, without having syphilis [1-3]. Positive reactions were found in tests, such as the VDRL-flocculation test, in which the main antigen is a phospholipid that could be extracted from beef heart, and therefore was named cardiolipin [4]. In a large retrospective study of these biologically false-positive plasmas, a group with a transient false positive reaction and a group with a chronic false positive reaction were defined [1]. In this latter group, the incidence of autoimmune disorders, notably SLE, was high, whereas transient positive reactions related to infectious diseases. These chronic false positive reactors are at present known as autoimmune anticardiolipin antibodies.

In 1952, a peculiar circulating anticoagulant was described in two SLE patients. This anticoagulant was able to prolong the prothrombin time, even when the plasma was diluted with normal plasma to correct for coagulation factor defiencies [5]. Surprisingly, it was not associated with a bleeding tendency. This anticoagulant was mainly found in SLE patients and therefore termed lupus anticoagulant [6]. Later, it became clear that lupus anticoagulant was not restricted to SLE patients alone, but that it was also found in association with certain drugs, infections, malignancies, and even in healthy individuals [7-10]. However, the name lupus anticoagulant is still in general use. The clinical importance of anticardiolipin and LAC-inducing antiphospholipid antibodies only became clear in the 1980s, when it was demonstrated that the presence of LAC is associated with thrombosis, thrombocytopenia and fetal loss [11].

In 1988, it became evident that anticardiolipin antibodies and antibodies with LAC activity are not necessarily the same antibodies, as was demonstrated by separating both types of antibodies from a single plasma [12-14].

Antiphospholipid antibodies

In 1990, it was shown that autoimmune anticardiolipin antibodies were not directed against negatively charged phospholipids directly, but against the phospholipid-binding protein β_2-glycoprotein I (β_2GPI)[15-17]. It was shown that β_2GPI, found in the fetal calf serum (FCS) used as blocking reagent in the anticardiolipin ELISA, contained the epitopes recognized by the antibodies.

Lateron, it was also shown that antibodies with LAC activity are dependent on the presence of either β_2GPI [18,19] or prothrombin [20], which are able to bind to negatively charged phospholipids, like cardiolipin and phosphatidylserine. Nowadays, antibodies against a variety of phospholipid-bound proteins have been described, such as high and low molecular weigth kininogens in complex with phosphatidylethanolamine [21-23], protein C [24], protein S [24-26], annexin V [27-29], factor XII [30-34] and plasminogen [35]. However, the relevance of these antibodies is unclear. Therefore, at present it is generally accepted that β_2GPI (see chapter 2) and prothrombin (see section prothrombin and anti-prothrombin antibodies) are the most important antigens in the antiphospholipid syndrome. It should be noted that the term antiphospholipid antibodies is quite confusing, since these autoantibodies are not directed against phospholipids. However, it is difficult to change these established names.

Detection of antiphospholipid antibodies

aCL are usually detected with an ELISA setup using immobilized cardiolipin as surface to bind the antigen β_2GPI, which is present in the fetal calf serum used for blocking [36]. At present, attempts to standardize this assay have not been very successful, although improvements have been made over the past years regarding background binding and quantification [37-40].

LAC activity can be detected using various *in vitro* phospholipid-dependent coagulation assays. The complexes of the LAC positive antibody and its antigen are able to bind with high affinity to the negatively charged phospholipid used in the assay, thereby competing with coagulation factors for these catalytic phospholipids. Since these phospholipids, needed as cofactor in coagulation, are 'consumed' by the antigen-antibody complexes, the result is a prolongation of the clotting times [41]. The criteria to detect LAC activity are as follows: (1) platelet-poor (platelet count should be less than 10×10^9/L) patient and normal plasma, prepared by a double centrifugation step at 2000 g for 10 min, should be used, (2) prolongation of clotting time should be present in at least one phospholipid-dependent coagulation assay, (3) the prolongation of clotting time should not disappear upon 1:1 dilution with pooled normal plasma, (4) phospholipid-dependency should be demonstrated by either neutralization of the effect upon the addition of increasing concentrations of phospholipids or enhancement of the prolongation in the presence of lower concentrations of phospholipids, (5) and finally the presence of a specific coagulation factor inhibitor should be excluded [41-44].

Many coagulation assays have been described sensitive to detect LAC activity. The activated partial thromboplastin time (aPTT), dilute Russell's viper venom time (dRVVT), the kaolin clotting time (KCT), and the dilute prothrombin time (dPT) are the most commonly used assays. Both the aPTT and the KCT activate the intrinsic route of coagulation, the dRVVT starts with the activation of factor X and factor IX, while the dPT activates the

extrinsic route (see section haemostasis). The sensitivity and the specificity of a test to detect LAC activity depends on the use of carefully prepared platelet poor plasma, the composition and concentration of phospholipids, the reagents, the device used, the procedure, and the type of antibody. No LAC test shows 100% specificity and sensitivity due to the heterogeneous nature of antiphospholipid antibodies. Therefore, the advised method to measure LAC activity is to perform at least two coagulation assays that start at different levels of the coagulation cascade, e.g. a sensitive aPTT and a dRVVT [44,45]. Although most of these tests are commercially available, the variation in the detection of LAC activity between laboratories is large [46]. Le Querrec *et al.* suggested a method that enables quantification of LAC activity. To do so, plasmas spiked with monoclonal antibodies against β_2GPI and prothrombin were used [47], which should improve the standardization of LAC activity in the future.

At present, anti-β_2GPI and anti-prothrombin antibodies can be measured by coating the proteins directly on irradiated ('high binding') ELISA plates. Anti-prothrombin antibodies are also detected by immobilizing phosphatidylserine vesicles, which are then able to bind prothrombin. However, since there is a large variability in these tests, they are mostly used for research purposes.

Prothrombin and anti-prothrombin antibodies

Prothrombin is a vitamin K-dependent plasma protein, which is synthesized in the liver [48,49]. It has a molecular weight of 72 kDa and its plasma concentration is 100 μg/mL. Prior to its secretion, ten glutamic acid residues at the N-terminus are specifically converted into γ-carboxyglutamic acid (Gla) by a vitamin K-dependent carboxylase. These Gla residues are essential for prothrombin to bind to negatively charged phospholipids. Besides the region in which the Gla residues are located, prothrombin consists of two kringle domains and a serine protease domain (catalytic domain). The kringle domains have a function in the interaction with other proteins, such as activated factor X, activated factor V, and factor XI [50-53]. Upon activation of the coagulation cascade, prothrombin can associate with activated factor X, activated factor V, phospholipids and calcium, together forming the prothrombinase complex (see section haemostasis)[48,49]. In this complex activated factor X is able to cleave prothrombin at positions Arg273-Thr274 and Arg322-Ile323. This cleavage results in the generation of fragment 1+2 and α-thrombin (fig. 1).

Antibodies to prothrombin can be found in the plasma of APS patients. However, the clinical relevance of these antibodies is not clear. Most *in vitro* studies have been focused on the effects of anti-β_2GPI antibodies. Now, the *in vitro* models developed to measure the effects of anti-β_2GPI antibodies are also used to determine the effects of anti-prothrombin antibodies. It has been shown that anti-prothrombin antibodies were able to increase the affinity of prothrombin for endothelial cells and immobilized phospholipids *in vitro* [54]. Recently, it became clear that prothrombin forms a bivalent complex with the anti-prothrombin antibodies [55,56], which is comparable to the mechanism of induction of LAC activity described for the interaction between β_2GPI-anti-β_2GPI antibodies (see chapter 2).

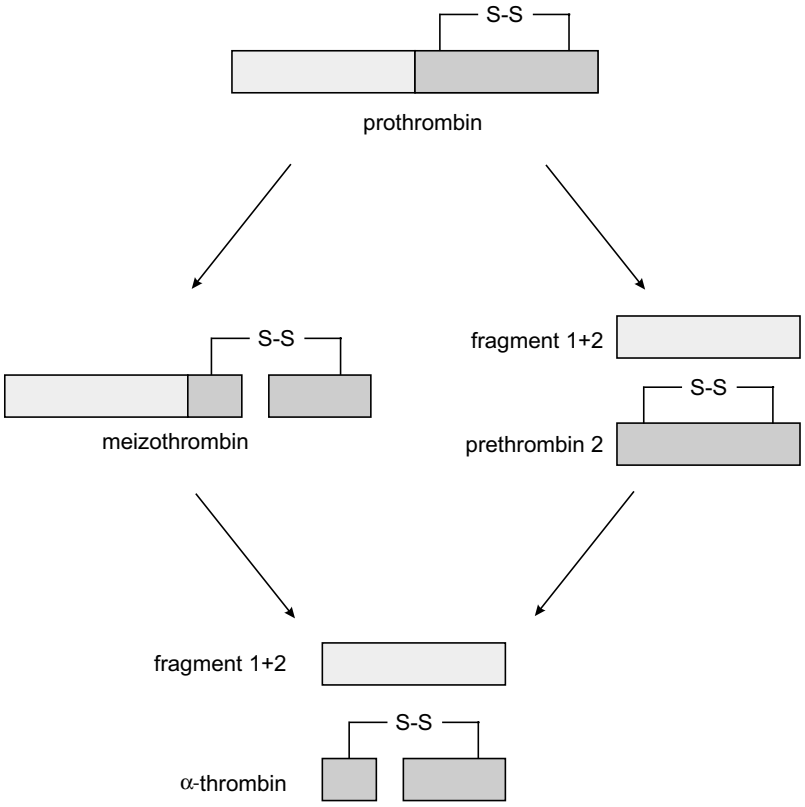

FIG. 1. **The activation of prothrombin to thrombin.** The proteolytic cleavage of prothrombin at Arg273-Thr274 by activated factor X results in fragment 1+2 and prethrombin 2. Further cleavage of prethrombin 2 at Arg322-Ile323 leads to the formation of α-thrombin. When prothrombin is first cleaved at Arg322-Ile323 and then at Arg273-Thr274, the formation of α-thrombin occurs via the intermediate meizo-thrombin.

Anti-β_2GPI versus anti-prothrombin antibodies

There is no consensus on the clinical relevance of anti-prothrombin and anti-β_2GPI antibodies. Three groups found an association between the presence of anti-prothrombin antibodies and venous thrombosis [57-59], and two of these studies reported also that the presence of anti-β_2GPI antibodies is associated with an increased risk for thrombosis [57,58]. A retrospective study by Pengo *et al.* and a meta-analysis by Galli *et al.* did not show an association between the presence of anti-prothrombin antibodies and thrombosis [60,61], although Pengo *et al.* did find a correlation between the presence of anti-β_2GPI antibodies and thrombosis [61]. However, the strongest association is found between LAC activity and thrombosis [57,62-64], suggesting that antibodies have to be ‘functional’ (LAC positive) in order to be pathogenic.

Haemostasis

Upon vessel injury, a haemostatic plug has to be formed. Plug formation starts with adhesion and aggregation of platelets onto the exposed connective tissue. During this process, the coagulation system becomes activated by the exposure of tissue factor (TF). This results via a cascade of proteolytic reactions in the formation of thrombin [65,66]. Thrombin then further converts fibrinogen to fibrin, which stabilizes the platelet plug. Furthermore, thrombin is able to activate platelets, endothelial cells, which have procoagulant properties, and the protein C system, which has an anticoagulant effect [67]. Under normal circumstances, the formation of a haemostatic plug only occurs outside the injured vessel. However, when a vessel wall is superficially injured, coagulation is activated inside a vessel. In the presence of risk factors for thrombosis, such as antiphospholipid antibodies, the prothrombotic process can proceed faster than it is inhibited by the physiological antithrombotic mechanisms, resulting in the development of a pathological thrombus inside the vessel.

Platelet adhesion and thrombus formation

Platelet adhesion to the damaged endothelium starts with the binding of plasma von Willebrand factor (vWF) to collagen, present in the exposed subendothelial layer. Subsequently, glycoprotein Ib (GPIb), which is located on the platelet surface as part of the glycoprotein Ib-V-IX complex, interacts with the vWF attached to the collagen fibers. This is a transient interaction, resulting in rolling of the platelet over the collagen surface. This is followed by the binding of the collagen receptors $\alpha_2\beta_1$ and glycoprotein VI (GPVI) on the platelet to collagen, which results in firm attachment and activation of the platelets. Activated $\alpha_{IIb}\beta_3$ is able to bind ligands, such as fibrinogen, vWF and fibronectin (all present in plasma), which then form a bridge between activated $\alpha_{IIb}\beta_3$ molecules, thereby connecting the platelets together. Further activation of the platelets is achieved by signal transduction via receptors, such as GPVI and the thrombin receptor PAR-1, resulting in the release of granule content. Adenosine 5'-diphosphate (ADP) and thromboxane are constituents of these granula, which are able to further activate the platelets, thereby promoting further and stable thrombus growth (fig. 2) [68-71].

Coagulation

The coagulation cascade can be activated by two pathways: the extrinsic pathway, which starts with the exposure of TF to the blood stream [72-74], and the intrinsic pathway, starting by the activation of factor XII [75-78]. Both pathways join each other in the tenase complex (fig. 3). Nowadays, it is generally accepted that *in vivo* TF is the only important activator of coagulation.

Upon vascular damage, tissue factor becomes exposed to the blood stream. Subsequently, activated factor VII, which is present in trace amounts in the circulation, interacts with TF, which starts the extrinsic route of coagulation. TF is also able to interact with factor VII, which can be proteolyzed to activated factor VII by activated factor X [79]. This TF-VIIa complex cleaves factor IX to activated factor IX, which in turn activates factor X in the presence of its cofactor, activated factor VIII. High concentrations of the TF-VIIa complex

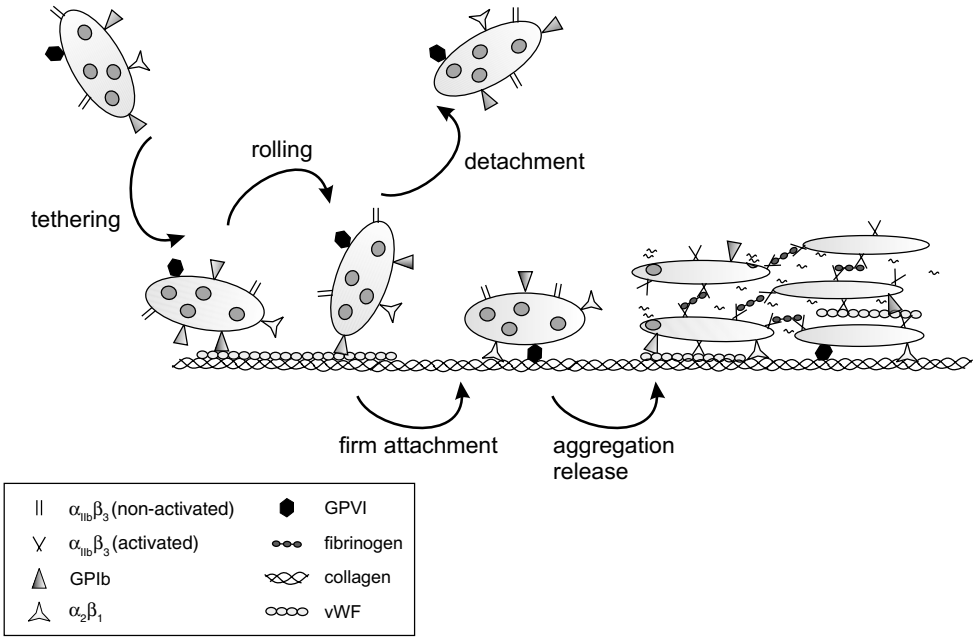

FIG. 2. **Platelet adhesion to collagen.** Collagen,exposed to the circulation, interacts with vWF. Then GPIb, located at the platelet surface, binds vWF, resulting in rolling of the platelets over the surface. Subsequently, the receptors $\alpha_2\beta_1$ and GPVI on the platelet interact with collagen, which results in firm attachment and activation. Activated $\alpha_{IIb}\beta_3$ then binds ligands as fibrinogen and vWF, thereby connecting the platelets together. Further activation of the platelets is achieved by release of the granule content, which activates other platelets.

can also directly activate factor X to activated factor X. This is followed by the assemblage of the prothrombinase complex, which is formed by activated factor X, its protein cofactor activated factor V, and prothrombin, resulting in the activation of prothrombin to thrombin [48]. Thrombin then converts fibrinogen into fibrin. Fibrin monomers will polymerize leading to the deposition of insoluble fibrin, which is able to stabilize the platelet plug [80]. Thrombin is also a potent activator of platelets and endothelial cells. Furthermore, it can activate positive and negative feedback loops (see section inhibition of coagulation). In the positive loops, thrombin activates factor XI and the protein cofactors factor V and factor VIII. Activated factor XI then can activate factor IX and this together with the cofactor activity of activated factor V and activated factor VIII, results in the formation of high concentrations of thrombin. For these reactions the presence of calcium and negatively charged phospholipids, as phosphatidylserine, are essential. Phosphatidylserine, which is in resting conditions located at the inner leaflet of the cell membrane, can become exposed to the circulation by a flip-flop mechanism that occurs after activation of platelets and endothelial cells [81-83].

The intrinsic route is activated by the exposure to negatively charged surfaces, such as glass, collagen and koalin. This results in the activation of factor XII in the presence of (pre)kallikrein and high molecular weight kininogen (HMWK). Subsequently, activated

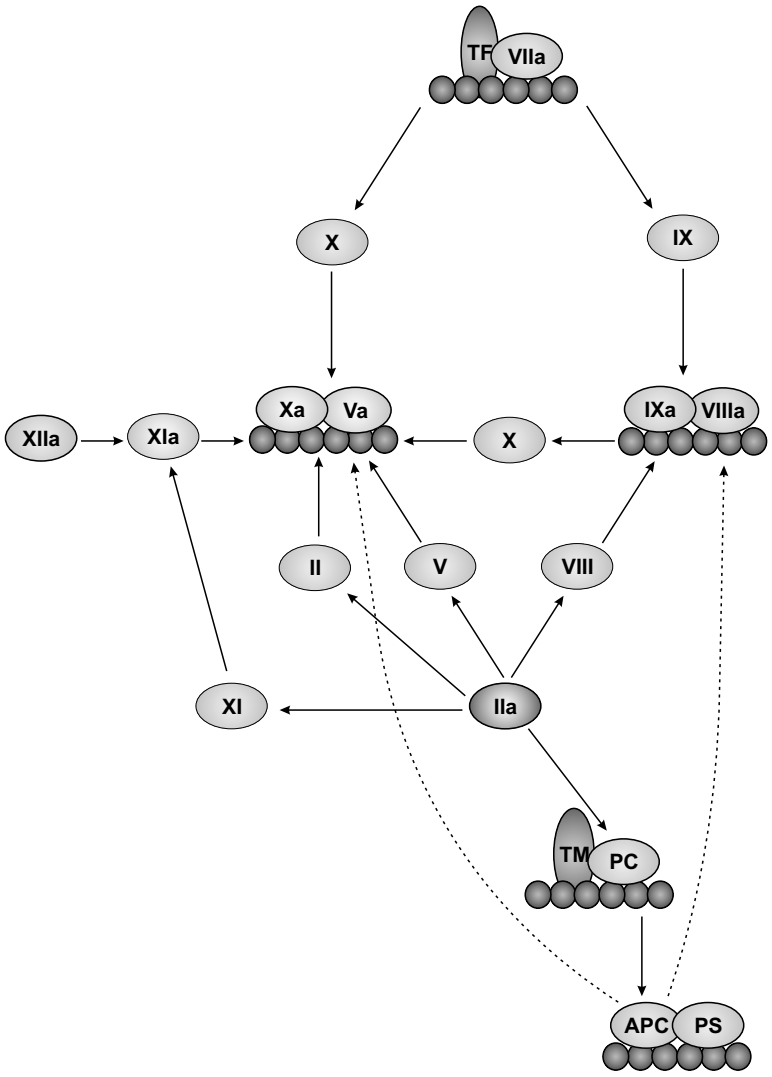

FIG. 3. **Schematic representation of the coagulation system.** Coagulation is initiated via the TF-VIIa complex or via the activation of factor XII. Most of the reactions require the presence of phospholipids and calcium. Coagulation factors are indicated by roman numericals, followed by 'a' in case of an activated coagulation factor. TF denotes tissue factor; PC, protein C; APC, activated protein C; PS, protein S; TM, thrombomodulin.

factor XII can activate factor XI, which can activate factor IX and thereby join the extrinsic coagulation cascade in the tenase complex.

Inhibition of coagulation

The coagulation cascade is controlled by protease inhibitors, of which tissue factor pathway inhibitor (TFPI) and anti-thrombin (AT) are the most important. TFPI inhibits the TF-VIIa complex [79,84] and AT [85] is able to inhibit all coagulation factors, except activated factor VII.

The haemostatic balance is also maintained by the protein C system [86-88]. In the presence of thrombomodulin (a transmembrane protein that is constitutively expressed on endothelial cells) thrombin activates, as a negative feedback loop, protein C to activated protein C (APC). This activation of protein C is facilitated by the endothelial cell protein C receptor (EPCR), which presents protein C to the thrombin/thrombomodulin complex. Then APC, together with its cofactor protein S, can inactivate the cofactors activated factor V and activated factor VIII and thereby inhibit coagulation. This mechanism is accelerated in the presence of negatively charged phospholipids.

Fibrinolysis

Fibrinolysis helps to restore vessel patency and is simultaneously activated with procoagulant pathways upon vascular damage [89,90]. Plasmin, the key enzyme in fibrinolysis, is formed by the activation of plasminogen. Most important enzymes for this activation are tissue-type plasminogen activator (tPA), of which the vascular endothelium is the major source, and urinary-type plasminogen activator (uPA), which is synthesized in the kidney and released into the urinary tract. Plasmin binds to fibrin and degradates a fibrin clot into small soluble fragments.

To regulate fibrinolysis, inhibitors are present in the plasma. At high concentrations of thrombin, factor XIII and thrombin-activatable fibrinolysis inhibitor (TAFI) are activated. Activated factor XIII stabilizes the fibrin clot by the formation of cross-links between the fibrin chains. TAFI inhibits fibrinolysis by interfering with the binding of plasminogen to fibrin, which results in a decrease in plasmin formation. Furthermore, plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2) are able to inhibit tPA and the active form of uPA directly.

An in vitro flow model

To study the interaction between platelets and the vessel wall under flow, similar to the condition present in blood vessels, a perfusion system, which became more sophisticated during the years, was developed [91-93]. Such a system consists of a perfusion chamber, an infusion pump with syringes, and tubes to connect the chambers and the syringes in the pump. At present, the mostly used perfusion chambers are the parallel plate chambers. The thrombogenic surface, which can be purified vessel wall adhesive proteins (e.g. collagen and fibronectin), cultured cells or endothelial cell matrix, is introduced on a coverslip, which is then placed on a polycarbonate knob. Subsequently, the chamber is placed into a 37°C waterbath and the blood is drawn over the surface at a defined flow rate by a syringe (fig. 4). After the perfusion, the platelets on the coverslip are fixed and stained. Finally, platelet deposition is evaluated with computer-assisted analysis.

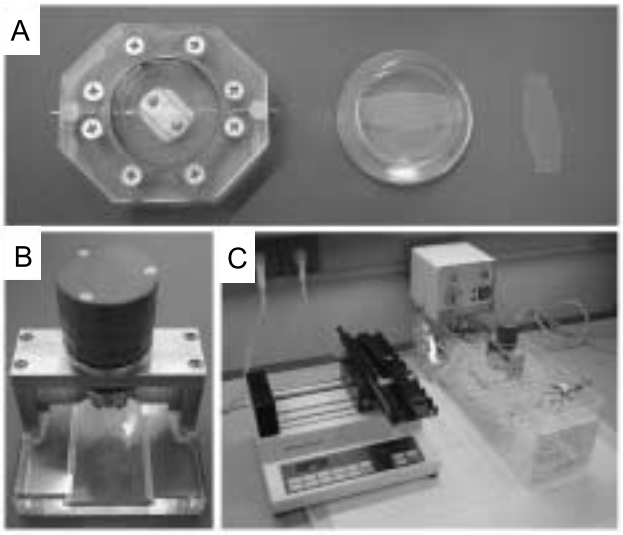

FIG. 4. **The perfusion system.** Coverslips, coated with a thrombogenic surface, are placed on a knob in a small perfusion chamber (*A*), which is placed in a holder (*B*). The perfusion chamber is then placed in a 37βC waterbath and exposed to flowing whole blood using a syringe pump (*C*).

The low-density lipoprotein receptor superfamily

The low-density lipoprotein (LDL) receptor family consists of several structurally related endocytic receptors. The first characterized family member was the LDL receptor, which function is, as far as known at present, restricted to lipoprotein metabolism. This receptor binds specifically LDL particles, after which the receptors cluster into coated pits in the plasma membrane. The final result of this process is the breakdown of LDL and the release of the lipids into the cytoplasm. Other members of this family include the LDL-receptor-related protein (LRP), megalin (also named glycoprotein-330), the very-low-density lipoprotein (VLDL) receptor and the apolipoprotein E receptor-2 (apoER2, also named LRP8). These receptors all are type I membrane proteins, carrying a single transmembrane domain, a small cytoplasmic tail and a large extracellular part at the amino terminus. This extracellular domain contains the ligand binding sites, which are formed by cysteine-rich ligand repeats of ~ 40 amino acids and three internal disulfide bonds. Furthermore, all receptors have epidermal growth factor (EGF) precursor homology domains, which are formed by EGF precursor type repeats and spacer regions containing YWTD motifs. These YWTD motifs are responsible for pH-dependent release of ligands in the endosomal compartments. Finally, all receptors have one or more copies of a NPxY motif in their cytoplasmic domain, which is important for directing the receptors into coated pits. In addition, a 600 kDa precursor of LRP is proteolytically cleaved, resulting in fragments of 515 kDa and 85 kDa, which are non-covalently associated. The LDL receptor, the VLDL receptor, and the apoER2 receptor contain a region rich in serine and threonine residues, which is extensively modified with O-linked sugars [94-97].

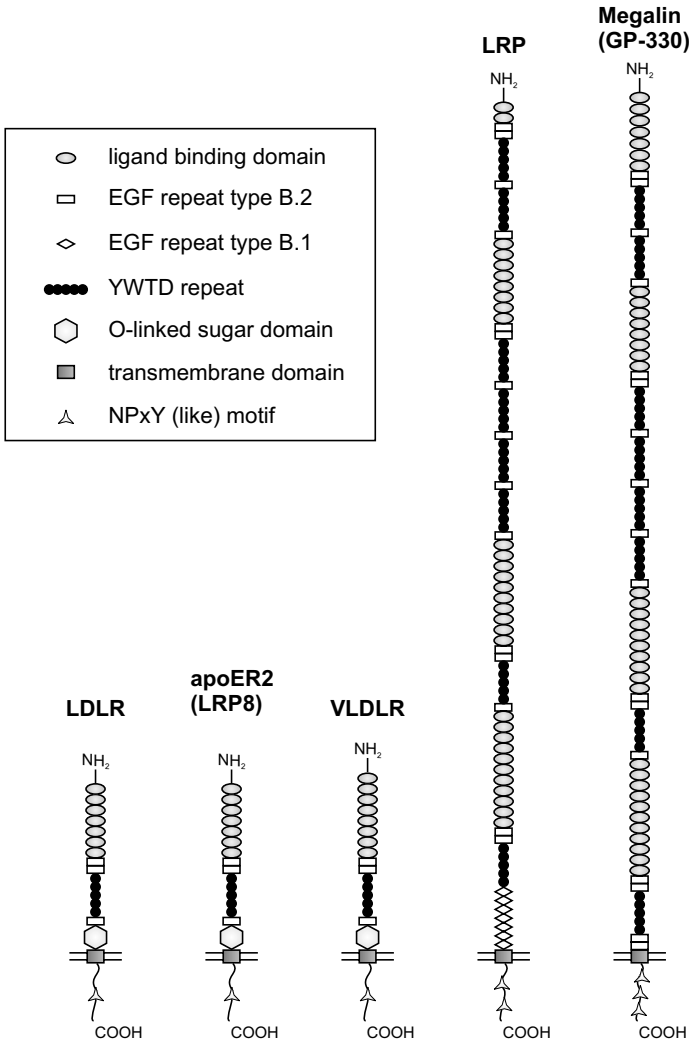

FIG. 5. **Schematic representation of the structure of members of the LDL-receptor superfamily.** These receptors all have an extracellular domain which consists of ligand binding repeats and EGF precursor homology domains formed by the EGF precursor type repeats and spacer regions containing YWTD (Tyr-Trp-Thr-Asp) motifs. The cytoplasmic tail contains one or more copies of a NPxY (Asn-Pro-x-Tyr) sequence. LDLR denotes LDL-receptor; VLDLR, VLDL-receptor; GP-330, glycoprotein-330.

A universal feature of the members of the LDL-receptor family is the ability of the 39 kDa receptor-associated protein (RAP) to inhibit the interaction of ligands with these receptors. Under physiolocal conditions, RAP is an endoplasmic reticulum (ER)-resident protein, that ensures safe passage through the secretory pathway by preventing premature interaction of ligands with members of the LDL-receptor family. Furthermore, RAP promotes proper folding of the receptors [98-100]. Below, three members of the LDL-receptor family, that are important for this study, will be discussed in more detail.

LRP

LRP is most abundantly expressed in the liver, placenta, lung and brain. Furthermore, it is present on various other cell types. Monocytes are the only blood cells expressing LRP. Originally, it was thought that LRP was involved in lipoprotein metabolism, but at present a broader physiological role is proposed. It has been shown to be involved in the regulation of lipases, proteases and their inhibitors (including coagulation factors), the metabolism of toxins and transduction of cellular signals. Moreover, LRP is essential for embryonic development, since $LRP^{-/-}$ knock-out mice die during gestation [96,97,101-103].

Megalin

Megalin is expressed abundantly on the apical surface of the epithelial cells of proximal tubules in the kidney. There it mediates uptake of carrier complexes of vitamins A and D, vitamin B_{12}-transcobalamin complex and glomerular filter polybasic drugs, such as nephro- and ototoxic aminoglycosides. It also has been shown that β_2GPI binds to megalin, and a role for β_2GPI in the cellular uptake of phosphatidylserine-exposing particles was proposed [104]. *In vitro*, it can bind many ligands that are also able to bind to LRP, such as lipoproteins, lipoprotein lipase, and urokinase. However, the *in vivo* function of these interactions is not yet clear. Furthermore, megalin-deficient mice are born alive, but most of them die perinatally from defects in the development of the forebrain [95,97,101,103,105].

ApoER2

ApoER2 is expressed mainly in the brain and testis. A splice variant, which lacks ligand binding domains 4 to 6 (named LRP8Δ4-6) can be found in platelets. It was shown that apolipoprotein E inhibits platelet aggregation via the activation of nitric oxide (NO) synthase. This suggests that the inhibitory effect of apolipoprotein E is mediated by LRP8Δ4-6 on platelets [106]. Furthermore, ApoER2 has an important role in the development of the brain. Mice deficient in ApoER2 and the VLDL-receptor have cerebellar dysplasia. This phenotype was identical to that of previously described knock-out mice, namely mice deficient in reelin or Disabled-1. Now, it is known that ApoER2 and the VLDL-receptor can directly bind reelin with high affinity, which is followed by the interaction of Disabled-1 with the NPxY motif in the cytoplasmic tail of these receptors. Subsequently, Disabled-1 is phosphorylated on tyrosine residues. In turn, Disabled-1 is thougth to activate nonreceptor tyrosine kinases of the Scr and Abl families, which further transmit signals essential for migration neurons [97,101,103,107].

Aim

The aim of this thesis is to gain insight into the mechanisms behind the development of thrombosis in the antiphospholipid syndrome. We have focused on β_2GPI and anti-β_2GPI antibodies. In chapter 2, a review about the current knowledge about this protein and its relation with the antiphospholipid syndrome is given. To investigate the effects of β_2GPI and anti-β_2GPI antibodies, we intended to develop an animal model for the antiphospholipid syndrome. We started to make mice transgenic for human β_2GPI. However, it appeared that in these mice the plasma levels of human β_2GPI were too low to enable use of these mice as a model for the antiphospholipid syndrome. Therefore, we focused on the thrombogenic properties of β_2GPI-anti-β_2GPI antibody complexes. To circumvent problems with making these complexes, we constructed chimeric dimers of β_2GPI and the dimerization domain of coagulation factor XI as a model for β_2GPI-anti-β_2GPI antibody complexes (chapter 3). These dimers were used to investigate their effects on platelet adhesion and aggregate formation in an *in vitro* flow system. To extent our observations with dimeric β_2GPI, the effects of anti-β_2GPI antibodies on platelet adhesion were also studied (chapter 4). To investigate whether anti-prothrombin antibodies have similar effects as anti-β_2GPI antibodies, these antibodies were also tested in the perfusion system (chapter 5). In chapter 6, the effects of dimers of β_2GPI and β_2GPI-anti-β_2GPI antibody complexes on platelet aggregation were described. In chapter 7, binding experiments using surface plasmon resonance (SPR) were performed to investigate the ability of dimeric β_2GPI to bind to different members of the LDL-receptor family. In chapter 8, the findings of these studies are discussed and placed in the context of the antiphospholipid syndrome.

References

1. Moore JE, Mohr CF. Biologically false positive serologic tests for syphilis: type, incidence, and cause. *J Am Med Association* 1952; 150: 467-473.
2. Moore JE, Lutz WB. The natural history of systemic lupus erythematosus. An approach to its study through chronic biologic false positive reaction. *J Chron Dis* 1955; 1: 297-316.
3. Laurell AB, Nilsson IM. Hypergammaglobinemia, circulating anticoagulant, and biological false positive Wasserman. *J Lab Clin Med* 1957; 49: 694-702.
4. Pangborn MC. A new serologically active phospholipid from beef heart. *Proc Soc Exp Biol* 1941; 48: 484-486.
5. Conley CB, Hartmann RC. A hemorrhagic disorder caused by circulating anticoagulant in patients with disseminated lupus erythematosus. *J Clin Invest* 1952; 31: 621-622.
6. Feinstein DI, Rapaport SI. Acquired inhibitors of blood coagulation. *Prog Hemostas Thromb* 1972; 1: 75-95.
7. Derksen RHWM, Kater L. Lupus anticoagulant: revival of an old phenomenon. *Clin Exp Rheumatol* 1985; 3: 349-357.
8. Colaco CB, Male DK. Anti-phospholipid antibodies in syphilis and a thrombotic subset of SLE: distinct profiles of epitope specificity. *Clin Exp Immunol* 1985; 59: 449-456.
9. Mueh JR, Herbst KD, Rapaport SI. Thrombosis in patients with the lupus anticoagulant. *Ann Intern Med* 1980; 92: 156-159.
10. Canoso RT, Hutton RA, Deykin DA. Chlorpromazine-induced inhibitor of blood coagulation. *Am J Hematol* 1977; 2: 183-191.
11. Hughes GRV. Thrombosis, abortion, cerebral disease an the lupus anticoagulant. *Br Med J* 1983; 287: 1088-1089.
12. Exner T, Sahman N, Trudinger B. Separation of anticardiolipin antibodies from lupus anticoagulant on a phospholipid-coated polystyrene column. *Biochem Biophys Res Commun* 1988; 155: 1001-1007.
13. McNeil HP, Chesterman CN, Krilis SA. Anticardiolipin antibodies and lupus anticoagulants comprise separate antibody subgroups with different phospholipid binding characteristics. *Br J Haematol* 1989; 73: 506-513.
14. Chamley LW, Pattison NS, McKay EJ. Separation of lupus anticoagulant from anticardiolipin antibodies by ion-exchange and gelfiltration chromatography. *Haemostasis* 1991; 21: 25-29.
15. Galli M, Comfurius P, Maassen C, Hemker HC, de Baets MH, van Breda-Vriesman PJC, Barbui T, Zwaal RFA, Bevers EM. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein factor. *Lancet* 1990; 335: 1544-1547.
16. McNeil HP, Simpson RJ, Chesterman CN, Krilis SA. Antiphospholipid antibodies are directed against a complex antigen that includes a lipid binding inhibitor of coagulation: β_2-glycoprotein I (apolipoprotein H). *Proc Natl Acad Sci USA* 1990; 87: 4120-4124.
17. Matsuura E, Igarashi Y, Fujimoto M, Ichikawa K, Koike T. Anticardiolipin cofactor(s)

and differential diagnosis of autoimmune disease. *Lancet* 1990; 336: 177-178.
18. Oosting JD, Derksen RHWM, Entjes HTI, Bouma BN, De Groot PhG. Lupus anticoagulant activity is frequently dependent on the presence of β_2-glycoprotein I. *Thromb Haemost* 1992; 67: 499-502.
19. Roubey RAS, Pratt CW, Buyon JP, Winfield JB. Lupus anticoagulant activity of autoimmune antiphospholipid antibodies is dependent upon beta 2-glycoprotein I. *J Clin Invest* 1992; 90: 1100-1104.
20. Bevers EM, Galli M, Barbui T, Comfurius P, Zwaal RFA. Lupus anticoagulant IgG's (LA) are not directed to phospholipids only, but to a complex of lipid-bound human prothrombin. *Thromb Haemost* 1991; 66: 629-632.
21. Sugi T, McIntyre JA. Autoantibodies to phosphatidylethanolamine (PE) recognize a kininogen-PE complex. *Blood* 1995; 86: 3083-3089.
22. Sugi T, McIntyre JA. Phosphatidylethanolamine induces specific conformational changes in the kininogens recognizable by antiphosphatidylethanolamine antibodies. *Thromb Haemost* 1996; 76: 354-360.
23. Sugi T, McIntyre JA. Autoantibodies to kininogen-phosphatidylethanolamine complexes augment thrombin-induced platelet aggregation. *Thromb Res* 1996; 84: 97-109.
24. Oosting JD, Derksen RHWM, Bobbink IWG, Hackeng TM, Bouma BN, De Groot PG. Antiphospholipid antibodies directed against a combination of phospholipids with prothrombin, protein C, or protein S: An explanation for their pathogenic mechanism. *Blood* 1993; 81: 2618-2625.
25. Sorice M, Arcieri P, Circella A, Misasi R, Lenti L, Di Nucci GD, Mariani G. Inhibition of protein S by autoantibodies in patients with acquired protein S deficiency. *Thromb Haemost* 1996; 75: 555-559.
26. D'Angelo A, Della Valle P, Crippa L, Pattarini E, Grimaldi LM, Vigano D. Brief report: autoimmune protein S deficiency in a boy with severe thromboembolic disease. *N Engl J Med* 1993; 328: 1753-1757.
27. Nakamura N, Shidara Y, Kawaguchi N, Azuma C, Mitsuda N, Onishi S, Yamaji K, Wada Y. Lupus anticoagulant autoantibody induces apoptosis in umbilical vein endothelial cells: Involvement of annexin V. *Biochem Biophys Res Commun* 1994; 205: 1488-1493.
28. Matsuda J, Gotoh M, Saitoh N, Gohchi K, Tsukamoto M, Yamamoto T. Anti-annexin antibody in the sera of patients with habitual fetal loss or preeclampsia. *Thromb Res* 1994; 75: 105-106.
29. Matsuda J, Saitoh N, Gohchi K, Gotoh M, Tsukamoto M. Anti-annexin V antibody in systemic lupus erythematosus patients with lupus anticoagulant and/or anticardiolipin antibody. *Am J Hematol* 1994; 47: 56-58.
30. Jones DW, Gallimore MJ, Winter M. Factor XII determinations and lupus anticoagulant. *Blood Coagul Fibrinol* 1997; 8: 531-532.
31. Gallimore MJ, Jones DW, Winter M. Factor XII determinations in the presence and absence of phospholipid antibodies. *Thromb Haemost* 1998; 79: 87-90.
32. Jones DW, Gallimore MJ, Harris SL, Winter M. Antibodies to factor XII associated with lupus anticoagulant. *Thromb Haemost* 1999; 81: 387-390.

33. Jones DW, Gallimore MJ, Mackie IJ, Harris SL, Winter M. Reduced factor XII levels in patients with the antiphospholipid syndrome are associated with antibodies to factor XII. *Br J Haematol* 2000; 110: 721-726.
34. Jones DW, Mackie IJ, Gallimore MJ, Winter M. Antibodies to factor XII and recurrent fetal loss in patients with the anti-phospholipid syndrome. *Br J Haematol* 2001; 113: 550-552.
35. Simmelink MJA. Interaction of antiphospholipid antibodies with the haemostatic process. *PhD Thesis, Utrecht University, The Netherlands* 2002; 57-68.
36. Harris EN, Gharavi AE, Boey ML, Patel BM, Mackworth-Young CG, Loizou S, Hughes GRV. Anticardiolipin antibodies: detection by radio-immunoassay and association with thrombosis in systemic lupus erythematosus. *Lancet* 1983; 2: 1211-1214.
37. Peaceman AH, Silver RK, MacGregor SN, Scool ML. Interlaboratory variation in antiphospholipid antibody testing. *Am J Obstet Gynecol* 1992; 144: 1780-1784.
38. Reber G, Arvieux J, Comby E, Degenne D, de Moerloose P, Sanmarco M, Potron G. Multicenter evaluation of nine commercial kits for the quantitation of anticardiolipin antibodies. *Thromb Haemost* 1995; 73: 444-452.
39. Tincani A, Allegri F, Sanmarco M, Cinquini M, Taglietti M, Balestrieri G, Koike T, Ichikawa K, Meroni PL, Boffa MC. Anticardiolipin antibody assay: a methodological analysis for a better consensus in routine determinations. *Thromb Haemost* 2001; 86: 583.
40. Tincani A, Balestrieri G, Allegri F, Cinquini M, Vianelli M, Taglietti M, Sanmarco M, Ichikawa K, Koike T, Meroni P, Boffa MC. Overview on anticardiolipin ELISA standardization. *J Autoimmun* 2000; 15: 195-197.
41. Arnout J. Antiphospholipid syndrome: diagnostic aspects of lupus anticoagulants. *Thromb Haemost* 2001; 86: 83-91.
42. Exner T, Triplett DA, Taberner D, Machin SJ. Guidelines for testing and revised criteria for lupus anticoagulants. *Thromb Haemost* 1991; 65: 320-322.
43. Brandt JT, Triplett DA, Alving B, Scharrer I. Criteria for the diagnosis of lupus anticoagulants: An update. *Thromb Haemost* 1995; 74: 1185-1190.
44. Greaves M, Cohen H, Machin SJ, Mackie I. Guidelines in the investigation and management of the antiphospholipid syndrome. *Br J Haematol* 2000; 109: 704-715.
45. Arnout J, Vermylen J. Lupus anticoagulants: mechanistic and diagnostic considerations. In: Khamashta MA, ed. *The Hughes Syndrome*. London: Springer-Verlag; 2000:225-237.
46. Arnout J, Meijer P, Vermylen J. Lupus anticoagulants testing in Europe: an analysis of results from the first european concerted action on thrombophilia (ECAT) survey using plasmas spiked with monoclonal antibodies against human β2-glycoprotein I. *Thromb Haemost* 1999; 81: 929-934.
47. Le Querrec A, Arnout J, Arnoux D, Borg JY, Caron C, Darnige L, Delahousse B, Reber G, Sié P. Quantification of lupus anticoagulants in clinical samples using anti-β_2GP1 and anti-prothrombin monoclonal antibodies. *Thromb Haemost* 2001; 86: 589.
48. Kalafatis M, Swords NA, Rand MD, Mann KG. Membrane-dependent reactions in blood coagulation: role of the vitamin K-dependent enzyme complexes. *Biochim*

Biophys Acta 1994; 1227: 113-129.
49. Esmon CT. Cell mediated events that control blood coagulation and vascular injury. *Annu Rev Cell Biol* 1993; 9: 1-26.
50. Deguchi H, Takeya H, Gabazza EC, Nishioka J, Suzuki K. Prothrombin kringle 1 domain interacts with factor Va during the assembly of prothrombinase complex. *Biochem J* 1997; 321: 729-735.
51. Kotkow KJ, Deitcher SR, Furie B, Furie BC. The second kringle domain of prothrombin promotes factor Va-mediated prothrombin activation by prothrombinase. *J Biol Chem* 1995; 270: 4551-4557.
52. Taneda H, Andoh K, Nishioka J, Takeya H, Suzuki K. Blood coagulation factor Xa interacts with a linear sequence of the kringle 2 domain of prothrombin. *J Biochem (Tokyo)* 1994; 116: 589-597.
53. Baglia FA, Badellino KO, Ho DH, Dasari VR, Walsh PN. A binding site for the kringle II domain of prothrombin in the apple 1 domain of factor XI. *J Biol Chem* 2000; 275: 31954-31962.
54. Rao LVM, Hoang AD, Rapaport SI. Mechanism and effects of the binding of lupus anticoagulant IgG and prothrombin to surface phospholipid. *Blood* 1996; 88: 4173-4182.
55. Simmelink MJA, Horbach DA, Derksen RHWM, Meijers JCM, Bevers EM, Willems GM, De Groot PG. Complexes of anti-prothrombin antibodies and prothrombin cause lupus anticoagulant activity by competing with the binding of clotting factors for catalytic phospholipids. *Br J Haematol* 2001; 113: 621-629.
56. Field SL, Chesterman CN, Dai YP, Hogg PJ. Lupus antibody bivalency is required to enhance prothrombin binding to phospholipid. *J Immunol* 2001; 166: 6118-6125.
57. Horbach DA, Van Oort E, Donders RCJM, Derksen RHWM, De Groot PG. Lupus anticoagulant is the strongest risk factor for both venous and arterial thrombosis in patients with systemic lupus erythematosus - Comparison between different assays for the detection of antiphospholipid antibodies. *Thromb Haemost* 1996; 76: 916-924.
58. Forastiero RR, Martinuzzo ME, Cerrato GS, Kordich LC, Carreras LO. Relationship of anti-β_2-glycoprotein I and antiprothrombin antibodies to thrombosis and pregnancy loss in patients with antiphospholipid syndrome. *Thromb Haemost* 1997; 78: 1008-1014.
59. Palosuo T, Virtamo J, Haukka J, Taylor PR, Aho K, Puurunen M, Vaarala O. High antibody levels to prothrombin imply a risk of deep venous thrombosis and pulmonary embolism in middle-aged men - A nested case-control study. *Thromb Haemost* 1997; 78: 1178-1182.
60. Galli M, Beretta G, Daldossi M, Bevers EM, Barbui T. Different anticoagulant and immunological properties of anti-prothrombin antibodies in patients with antiphospholipid antibodies. *Thromb Haemost* 1997; 77: 486-491.
61. Pengo V, Biasolo A, Brocco T, Tonetto S, Ruffatti A. Autoantibodies to phospholipid-binding plasma proteins in patients with thrombosis and phospholipid-reactive antibodies. *Thromb Haemost* 1996; 75: 721-724.
62. D'Angelo A, Safa O, Crippa L, Garlando A, Sabbadini MG, D'Angelo SV. Relationship

of lupus anticoagulant, anticardiolipin, anti-β_2-GPI and anti-prothrombin autoantibodies with history of thrombosis in patients with the clinical suspicion of APA-syndrome. *Thromb Haemost* 1997; 78: 967-968.
63. Wahl DG, Guillemin F, de Maistre E, Perret C, Lecompte T, Thibaut G. Risk for venous thrombosis related to antiphospholipid antibodies in systemic lupus erythematosus-a meta-analysis. *Lupus* 1997; 6: 467-473.
64. Wahl DG, Guillemin F, de Maistre E, Perret-Guillaume C, Lecompte T, Thibaut G. Meta-analysis of the risk of venous thrombosis in individuals with antiphospholipid antibodies without underlying autoimmune disease or previous thrombosis. *Lupus* 1998; 7: 15-22.
65. Macfarlane RG. An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. *Nature* 1964; 202: 498-499.
66. Davie EW, Ratnoff OD. Waterfall sequence for intrinsic blood clotting. *Science* 1964; 145: 1310-1312.
67. Griffin JH. Blood coagulation - The thrombin paradox. *Nature* 1995; 378: 337-338.
68. Sixma JJ, Van Zanten GH, Huizinga EG, van der Plas RM, Verkleij M, Wu YP, Gros P, De Groot PG. Platelet adhesion to collagen: an update. *Thromb Haemost* 1997; 78: 434-438.

69. Ruggeri ZM. von Willebrand factor. *J Clin Invest* 1997; 99: 559-564.
70. Jackson SP, Mistry N, Yuan Y. Platelets and the injured vessel wall - "Rolling into action". Focus on glycoprotein Ib/V/IX and the platelet cytoskeleton. *Trends Cardiovasc Med* 2001; 10: 192-197.
71. Dopheide SM, Yap CL, Jackson SP. Dynamic aspects of platelet adhesion under flow. *Clin Exp Pharmacol Physiol* 2002; 28: 355-363.
72. Davie EW, Fujikawa K, Kisiel W. The coagulation cascade: Initiation, maintenance, and regulation. *Biochemistry* 1991; 30: 10363-10370.
73. Rapaport SI, Rao LVM. The tissue factor pathway: How it has become a ''prima ballerina". *Thromb Haemost* 1995; 74: 7-17.
74. Nemerson Y. The tissue factor pathway of blood coagulation. *Semin Hematol* 1992; 29: 170-176.
75. Meijers JCM, McMullen BA, Bouma BN. The contact activation proteins: a structure/function overview. *Agents Actions Suppl* 1992; 38: 219-230.
76. Heimark RL, Kurachi K, Fujikawa K, Davie EW. Surface activation of blood coagulation, fibrinolysis and kinin formation. *Nature* 1980; 286: 456-460.
77. Wachtfogel YT, De La Cadena RA, Colman RW. Structural biology, cellular interactions and pathophysiology of the contact system. *Thromb Res* 1993; 72: 1-21.
78. Colman RW, Schmaier AH. Contact system: a vascular biology modulator with anticoagulant, profibrinolytic, antiadhesive, and proinflammatory attributes. *Blood* 1997; 90: 3819-3843.
79. Morrissey JH. Tissue factor: an enzyme cofactor and a true receptor. *Thromb Haemost* 2001; 86: 66-74.
80. Mosesson MW. The roles of fibrinogen and fibrin in hemostasis and thrombosis. *Semin Hematol* 1992; 29: 177-188.
81. Zwaal RF, Comfurius P, Bevers EM. Lipid-protein interactions in blood coagulation.

Biochim Biophys Acta 1998; 1376: 433-453.
82. Sims PJ, Wiedmer T. Unraveling the mysteries of phospholipid scrambling. *Thromb Haemost* 1999; 86: 266-275.
83. Zwaal RFA, Comfurius P, van Deenen LLM. Membrane asymmetry and blood coagulation. *Nature* 1977; 268: 358-360.
84. Broze GJ, Jr. Tissue factor pathway inhibitor. *Thromb Haemost* 1995; 74: 90-93.
85. Rosenberg RD, Rosenberg IR. Natural anticoagulant mechanisms. *J Clin Invest* 1984; 74: 1-6.
86. Esmon CT. Regulation of blood coagulation. *Biochim Biophys Acta* 2000; 1477: 349-360.
87. Esmon CT. The protein C anticoagulant pathway. *Arterioscler Thromb* 1992; 12: 135-145.
88. Dahlback B. The protein C anticoagulant system: inherited defects as basis for venous thrombosis. *Thromb Res* 1995; 77: 1-43.
89. Collen D. The plasminogen (fibrinolytic) system. *Thromb Haemost* 1999; 182: 259-270.
90. Lijnen HR. Elements of the fibrinolytic system . *Ann N Y Acad Sci* 2001; 936: 226-236.
91. Baumgartner HR. The role of blood flow in platelet adhesion, fibrin deposition, and formation of mural thrombi. *Microvasc Res* 1973; 5: 167-179.
92. Sakariassen KS, Aarts PAMM, De Groot PhG, Houdijk WPM, Sixma JJ. A perfusion chamber developed to investigate platelet interaction in flowing blood with human vessel wall cells, their extracellular matrix and purified components. *J Lab Clin Med* 1983; 102: 522-535.
93. Sixma JJ, De Groot PG, van Zanten H, IJseldijk M. A new perfusion chamber to detect platelet adhesion using a small volume of blood. *Thromb Res* 1998; 92: S43-S46.
94. Strickland DK, Gonias SL, Argraves WS. Diverse roles for the LDL receptor family. *Trends Endocrinol Metab* 2002; 13: 66-74.
95. Willnow TE. The low density lipoprotein receptor gene family: multiple roles in lipid metabolism. *J Mol Med* 1999; 77: 306-315.
96. Neels JG, Horn IR, van den Berg BMM, Pannekoek H, Van Zonneveld A-J. Ligand-receptor interactions of the low density lipoprotein receptor-related protein, a multi-ligand endocytic receptor. *Fibrinol Proteol* 1998; 12: 219-240.
97. Willnow TE, Nykjær A, Herz J. Lipoprotein receptors: new roles for ancient proteins. *Nat Cell Biol* 1999; 1: E157-E162.
98. Bu G. The roles of receptor-associated protein (RAP) as a molecular chaperone for members of the LDL receptor family. *Int Rev Cytol* 2001; 209: 79-116.
99. Bu G, Schwartz AL. RAP, a novel type of ER chaperone. *Trends Cell Biol* 1998; 8: 272-276.
100. Bu G, Marzolo MP. Role of RAP in the biogenesis of lipoprotein receptors. *Trends Cardiovasc Med* 2000; 10: 148-155.
101. Herz J, Bock HH. Lipoprotein receptors in the nervous system. *Annu Rev Biochem* 2002; 71: 405-435.
102. van der Geer P. Phosphorylation of LRP1: regulation of transport and signal

transduction. *Trends Cardiovasc Med* 2002; 12: 160-165.
103. Herz J, Gotthardt M, Willnow TE. Cellular signalling by lipoprotein receptors. *Curr Opin Lipidol* 2000; 11: 161-166.
104. Moestrup SK, Schousboe I, Jacobsen C, Leheste JR, Christensen EI, Willnow TE. β_2-glycoprotein I (apolipoprotein H) and β_2-glycoprotein I-phospholipid complex harbor a recognition site for the endocytic receptor megalin. *J Clin Invest* 1998; 102: 902-909.
105. Gliemann J. Receptors of the low density lipoprotein (LDL) receptor family in man. Multiple functions of the large family members via interaction with complex ligands. *Biol Chem* 1998; 379: 951-964.
106. Riddell DR, Vinogradov DV, Stannard AK, Chadwick N, Owen JS. Identification and characterization of LRP8 (apoER2) in human blood platelets. *J Lip Res* 1999; 40: 1925-1930.
107. Gleeson JG, Walsh CA. Neuronal migration disorders: from genetic diseases to developmental mechanisms. *Trends Neurosci* 2000; 23: 352-359.

Chapter 2

β_2-glycoprotein I
- a key player in the antiphospholipid syndrome -

Bianca C. H. Lutters[1,3], Philip G. de Groot[1,3], and Ronald H.W. M. Derksen[2]

Departments of [1]Haematology and [2]Rheumatology and Clinical Immunology, University Medical Center, Utrecht and [3]Institute of Biomembranes, Utrecht University, The Netherlands.

Israelian Medical Association Journal 4(suppl), 958-962, 2002

The plasma protein β_2-glycoprotein I (β_2GPI) was first described by Schultze *et al.* in 1961 [1], but world-wide interest in this molecule, with unknown physiological relevance, only occurred after its identification as a major epitope in tests that are widely used for the detection of so-called antiphospholipid antibodies (aPL). These antibodies had been identified as serological markers of a risk for thrombo-embolic complications, with clinical manifestations in venous, arterial, and placental vascular beds (the antiphospholipid syndrome, APS). Many research groups, including ourselves, spent much effort in getting more knowledge on the molecule β_2GPI itself, and to get insight into mechanisms by which antibodies directed to β_2GPI might promote thrombosis. In this review we will discuss current knowledge on β_2GPI and the potential pathophysiological role of anti-β_2GPI antibodies in relation to thrombosis.

The protein β_2-glycoprotein I

β_2-glycoprotein I, also known as apolipoprotein H, is a member of the short consensus repeat (SCR) or complement control protein (CCP) superfamily [2]. Short consensus repeat domains are present in many proteins functioning in the complement system [3] and consist of about 60 residues and two fully conserved disulfide bonds. Sequence homology among SCR domains ranges between 20 and 40 %. β_2GPI has a molecular weight of 42 kDa and consists of 326 amino acids [2]. The glycoprotein is built up out of five SRC domains, of which the fifth domain is aberrant. This domain has an ' extra' six-residue insertion and a C-terminal extension of 19 amino acids, which is C-terminally cross-linked by an additional C-terminal disulfide bond.

Many studies conclude that the aberrant fifth domain of β_2GPI is essential for binding of β_2GPI to negatively charged phospholipids [4-8]. Both inhibition studies in which synthetic peptides were used and mutagenesis studies clearly show that the sequence Cys281-Cys288 is very important in phospholipid binding [4,5]. Interest in another region in the fifth domain was raised by the finding that β_2GPI, cleaved between Lys317 and Thr318, is unable to bind negatively charged phospholipids [6]. More recently, it was shown that a polymorphism in which Ser316 is mutated into Trp, results in a form of β_2GPI which has lost its ability to bind to negatively charged phospholipids [7]. Furthermore, site-directed mutagenesis showed that the hydrophobic sequence at position 313-316 is crucial for phospholipid binding [8].

The elucidation of the crystal structure of β_2GPI in 1999 [9,10] nicely brought these observations together. As shown in figure 1, the crystal structure of β_2GPI reveals an extended chain of SCR domains in a fishhook-like form. The aberrant parts of the fifth domain (the 6 amino acid insertion and the 19 amino acid extension) are essential for phospholipid binding. The fifth domain constitutes a 2000Å large patch of 14 positively charged amino acids that can have electrostatic interactions with anionic headgroups of phospholipids. Probably, this interaction is followed by insertion of the flexible hydrophobic loop (Ser311-Lys317) into the phospholipid bilayer [9]. This positions Trp316 at the interface of the acyl chains and the phosphate headgroups of phospholipids, thereby anchoring the molecule to the membrane, a mechanism that is common for membrane binding proteins (figure 2). Studies by Lee *et al.* [11] suggest that also the first domain interacts with phospholipids. They reported a rapid binding of the fifth domain of β_2GPI to phosphatidylserine (PS) at isotonic and low ionic strength, which is followed by binding of the first domain of β_2GPI to the negatively charged phospholipids at low ionic strength. At this moment, it is not clear if the β_2GPI molecule is

able to bind with both the N- and the C-terminus to one membrane or if the β_2GPI molecule forms a bridge between two membranes. The latter possibility seems more likely, since the β_2GPI molecule is due to the presence of rather small linker regions probably a rather rigid molecule.

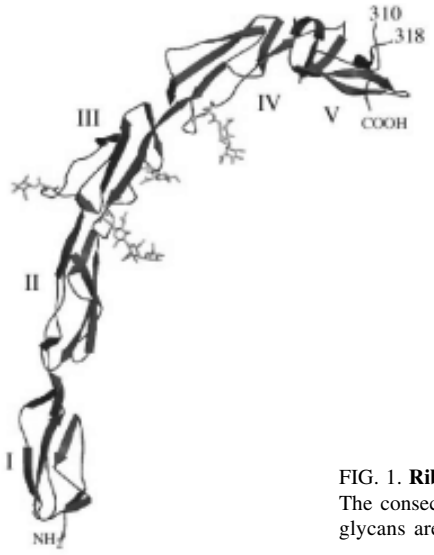

FIG. 1. **Ribbon drawing of the structure of β_2GPI.** The consecutive domains are labeled I to V and the glycans are depicted with ball-and-stick models.

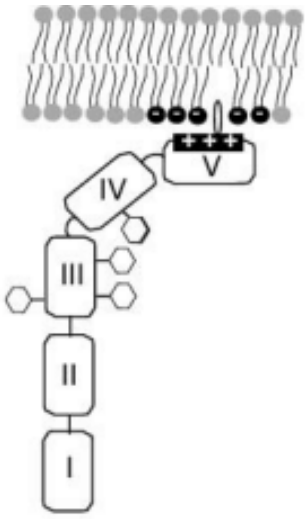

FIG. 2. **Cartoon for the proposed mechanism of binding of β_2GPI to a phospholipid surface.** The positively charged patch in the fifth domain is indicated with '+', the negatively charged phospholipids are depicted with '-'. The putative membrane insertion loop Ser311-Lys317 is shown to insert into the membrane. Position of N-glycans are indicated with hexagons.

The physiological role of β_2GPI

Compared to extensive knowledge on the structure of β_2GPI, we know very little about the physiological functions of this molecule. In most human beings the protein circulates in plasma at a concentration of about 200 µg/mL, of which approximately 40% is bound to lipoproteins [12]. However, complete absence of β_2GPI is not accompanied by apparent abnormalities [13]. Also in β_2GPI null mice no clear abnormalities have been found thusfar, although there is some discussion whether reproductivity and thrombin generation are somewhat impaired [14]. These observations seem to indicate that β_2GPI is a compound without any clear function.

As β_2GPI has been identified as a constituent of chylomicrons, very low density and high-density lipoproteins, it has been suggested that the molecule may be involved in lipoprotein metabolism [12]. The term apolipoprotein H was given to this compound when the suggestion had been given that β_2GPI activates lipoprotein lipase [15]. Recently, it was reported that β_2GPI inhibits both low-density lipoprotein oxidation and cholesterol accumulation by macrophages *in vitro* [16]. This inhibition of cholesterol accumulation was a result of a reduction of cholesterol influx and an increase in cholesterol efflux. This suggests that β_2GPI may play an important role in the prevention of atherosclerosis.

It has frequently been suggested that β_2GPI can have a role as neutralizer of coagulation as the protein can bind to negatively charged molecules, which are well-known catalysts of coagulation [17]. *In vitro* data suggest that β_2GPI can inhibit ADP-induced aggregation of platelets [18] and that β_2GPI may act as a modulator of glycoprotein $\alpha_{IIb}\beta_3$-dependent functions of platelet microparticle membranes [19], as this molecule can reduce the immunoreactivity of glycoprotein $\alpha_{IIb}\beta_3$ by binding to these membranes. Furthermore, β_2GPI can bind C4b-binding protein (C4BP), which is a physiological carrier of protein S. Unbound protein S in plasma has a role as cofactor in the inactivation of factor Va and factor VIIIa by activated protein C (APC). Therefore, binding of β_2GPI to C4BP increases the plasma levels of free protein S [20]. Other *in vitro* observations are that β_2GPI can inhibit tissue factor activity, the contact activation pathway of coagulation [21], and the activity of activated protein C activity [22]. Apart from the latter observation, which suggests a procoagulant effect of the compound, most *in vitro* data suggest that β_2GPI has anticoagulant effects. Reported effects of β_2GPI on coagulation generally suppose competition between β_2GPI and other proteins that are able to bind to negatively charged phospholipids. However, although β_2GPI does bind to negatively charged surfaces, β_2GPI does so with an affinity that is far below that of many other phospholipid binding proteins [23,24]. This suggests that the relevance of these *in vitro* observations for the situation *in vivo* is very disputable [21].

A physiological function completely different from that in coagulation has recently been suggested, namely removal of apoptotic cells [25-27]. It was found that β_2GPI associates with liposomes containing negatively charged phospholipids. These phospholipids are normally only found in the interior of cells but become exposed on the outside of cells when these undergo apoptosis [25]. It was shown that β_2GPI inhibits the procoagulant activity of liposomes and enhances uptake of these particles by macrophages [27].

β_2GPI as the antigen in the antiphospholipid syndrome

The antiphospholipid syndrome (APS) was defined in the mid-eighties based upon associations found between the persistent presence of the lupus anticoagulant (LAC) and anticardiolipin antibodies (aCL) with thrombosis, recurrent pregnancy loss and thrombocytopenia [28]. Originally, this syndrome was defined in patients with systemic lupus erythematosus (SLE), the disorder with the highest frequency of aPL. However, APS was soon recognized in patients without other overt clinical manifestations of a systemic autoimmune disease (primary APS). The term LAC refers to antibodies that prolong phospholipid-dependent coagulation assays, whereas aCL are detected in an enzyme-linked immunosorbent assay (ELISA) with the negatively charged phospholipid cardiolipin coated onto microtiter plates. In 1990 three groups independently reported that sera from patients with APS contain aCL that are not directed against phospholipids per se, but that a cofactor was involved, namely the plasma protein β_2GPI [29-31]. Soon after this discovery it was shown that β_2GPI was also essential in enabling aPL to prolong clotting times in phospholipid dependent coagulation tests [32]. These findings were the starting point for many new findings on structure and function of this protein and for research on the pathogenic potentials of anti-β_2GPI antibodies.

That β_2GPI is indeed an important antigen in APS is supported by experiments with animal models. Gharavi *et al.* induced aPL in mice and rabbits by immunization with purified human β_2GPI [33]. This was confirmed by Blank *et al.* [34]. This group found that immunization of mice with β_2GPI resulted in the production of high levels of aCL. Three months after a boost injection with β_2GPI, the mice had a prolonged activated partial thromboplastin time (aPTT), thrombocytopenia and an increase in fetal resorption (all features of APS). Data obtained in an experimental *in vivo* mouse model in which thrombus formation upon an artificially damaged vessel wall can be studied, clearly suggest that anti-β_2GPI antibodies induced by immunization have pathogenic potential as these increase the size and the persistence of formed thrombi [35].

Several studies have been done to elucidate where anti-β_2GPI antibodies bind on the β_2GPI molecule. Two studies, using recombinant domain-deleted β_2GPI and surface plasmon resonance respectively, point to domain 1 as the major target of anti-β_2GPI antibodies [36,37]. However, several groups identified different regions of β_2GPI, scattered all over the molecule, as possible epitopes for anti-β_2GPI antibodies [38-41]. Taking into account the crystal structure of β_2GPI, it seems unlikely that the third and fourth domain are candidates for the location of the antibody binding site, as these domains are heavily glycosylated and therefore poorly available for anti-β_2GPI antibodies. Since the fifth domain is the phospholipids-binding domain, it seems unlikely that β_2GPI-anti-β_2GPI antibody complexes will be able to bind to phospholipids when the anti-β_2GPI antibody binds to sequences in the fifth domain close to those involved in binding to phospholipids, making also this domain a poor candidate. The crystal structure suggests that the availability for antibody binding of both the first and second domain is very good.

Experiments performed with synthetic peptides that mimic epitopes on different regions of β_2GPI, namely the link between domain 1 and 2, domain 3 and domain 4, seem very informative. These three peptides react specificically with three different monoclonal anti-β_2GPI antibodies and were found using a phage display library. It was shown that experimental APS, induced by immunization with the anti-β_2GPI antibodies, can be prevented by the

administration of the synthetic peptide that reacts with the corresponding anti-β_2GPI antibody [41]. Furthermore, the notion that the structure of these 'protective' peptides have homology with membrane proteins of several bacteria (such as *neisseria gonorrhoeae* and *pseudomonas aeroginosa*) and viruses (such as *cytomegalovirus* and *human immunodeficiency virus*) suggests that infection and molecular mimicry may be important in the induction of anti-β_2GPI antibodies in man [42]. This is supported by recent findings that one can induce 'pathogenic' aPL in mice upon immunization with a *cytomegalovirus*-derived peptide, which shares homology with the phospholipid-binding site of β_2GPI [43]. However, the sequence in the β_2GPI molecule that shares homology with the *cytomegalovirus* peptide is hidden inside the molecule and not exposed to plasma, suggesting that β_2GPI, to be accessible for the antibodies, has to undergo a major conformational change or has to be denatured.

Pathophysiological effects of β_2GPI-anti-β_2GPI antibody complexes

Recognition of the importance of β_2GPI as antigen in APS has stimulated research to elucidate mechanisms by which anti-β_2GPI antibodies might induce thrombosis. Interesting observations include the potential of anti-β_2GPI antibodies to activate endothelial cells and platelets. It has been reported that anti-β_2GPI antibodies can upregulate the expression of adhesion molecules on endothelial cells, increase the secretion of cytokines by endothelial cells and up-regulate the production of prostacyclin [44]. In studies by Blank *et al.* synthetic peptides reacting with monoclonal anti-β_2GPI antibodies could prevent endothelial cell activation in experimental APS [41], and monoclonal anti-β_2GPI antibodies increased adhesion of leucocytes to vessel walls in another animal model [43]. In 1981, it was described that LAC positive IgG from a patient with APS reduced prostacyclin synthesis in bovine endothelial cells [45], and since then many studies on effects of aPL on prostacyclin synthesis in endothelial cells and thromboxane synthesis in platelets have been performed [46]. Results frequently suggested that aPL can indeed alter the balance between the antithrombotic effects of prostacyclin and the prothrombotic effects of thromboxane synthesis in such a way that the net effect might be prothrombotic, although results were frequently contradictory [46]. In a careful study by Hasselaar *et al.* [47], a disturbed balance between prostacyclin and thromboxane production was only found in some APS patients, and if present, this disbalance was unrelated to a history of thrombosis. However, studies on effects of aPL on prostaglandins and thromboxane have not been performed specifically with anti-β_2GPI antibodies. In this respect, the observation by Forastiero *et al.* that patients with APS and moderate to increased levels of anti-β_2GPI antibodies have high urinary levels of 11-dehydro-thromboxane B_2 (a stable metabolite of thromboxane A_2) compared with controls can be important [48] and may stimulate further research. Further, several groups have shown that aPL have a stimulatory effect on platelet aggregation [49-51]. Already in 1993, it was shown that monoclonal anti-β_2GPI were able to induce aggregation in the presence of subthreshold concentrations of ADP [52].

Recently, another effect of anti-β_2GPI antibodies was proposed [53]. It was found that incubation of human umbilical vein endothelial cells (HUVEC) with antiphospholipid antibodies resulted in accumulation of these antibodies at the cell surface and within late endosomes. However, at this moment it is not clear if these antibodies bind to the phospholipids directly, or if they bind only to the cells in the presence of β_2GPI. The authors [53] also showed that incubation of HUVEC with antiphospholipid antibodies results in a redistribution of the

cation-independent mannose-6-phosphate receptor (CI-M6PR) from the Golgi apparatus to late endosomes. This redistribution of CI-M6PR is expected to interfere with its normal function, namely the transport of lysosomal proteins into lysosome, followed by their secretion. It was suggested that the lysosomal proteins might activate endothelial cells, resulting in an inflammatory effect and a procoagulant state.

An important question is how anti-β_2GPI antibodies might have these cellular effects. In our current hypothesis, the notion that dimerization of β_2GPI occurs after interaction with an antibody and that this is accompanied a significant increase in the affinity of β_2GPI for negatively charged surfaces (compared to β_2GPI) is a central theme [23,54,55]. It is now clear that the high affinity of β_2GPI-anti-β_2GPI complexes for negatively charged phospholipids is the explanation for the prolongation in clotting times that anti-β_2GPI antibodies induce in functional *in vitro* assays, an abnormality that can be corrected by addition of extra phospholipids to the test system (viz. explain LAC activity) [56]. Obviously, β_2GPI-anti-β_2GPI complexes, in contrast to β_2GPI, can effectively compete with clotting factors for binding to phospholipids. Although our hypothesis on the importance of dimerization of β_2GPI to obtain pathophysiological relevance was recently challenged by the notion that Fab-fragments also enhance β_2GPI binding when measured with surface plasmon resonance [57], the hypothesis is strongly supported by our recent observations with constructs of dimeric β_2GPI that consist of two molecules β_2GPI and the dimerization motif of coagulation factor XI [58]. These dimers have a significantly increased affinity for negatively charged phospholipids compared to plasma-derived β_2GPI, have LAC activity and bind to membranes of activated platelets [58]. It is tempting to speculate that competition for binding to appropriate surfaces between β_2GPI-anti-β_2GPI complexes and other phospholipid binding proteins is important to explain at least some of the before mentioned pathophysiological conditions related to anti-β_2GPI antibodies. In this respect, presence of anti-β_2GPI antibodies could be considered as being ‘gain-of function’ proteins for β_2GPI.

More attention should be given to the two cellular receptors that have been described for β_2GPI, namely annexin II and megalin. Annexin II is an endothelial cell receptor for tissue-type plasminogen activator (tPA) and plasminogen [59], whereas megalin is identified as the renal clearance receptor for β_2GPI, since high concentrations of β_2GPI were detected in the urine of mice with a disrupted megalin gene [60]. Dimerization of such receptors by β_2GPI-anti-β_2GPI complexes might very well explain cellular activation. It also has been suggested that anti-β_2GPI antibodies can activation of cells via a Fc-receptor-mediated mechanism [61]. However, such mechanism is contradicted by the finding that $F(ab)_2$ fragments of anti-β_2GPI antibodies (with LAC activity) do promote thrombus formation in a hamster thrombosis model [62].

Conclusions

Although knowledge on β_2GPI and anti-β_2GPI antibodies expanded tremendously over the past decade we are still far from knowing the pathophysiological role of these proteins in the related thrombotic tendency. Given the heterogeneity of APS, both clinically and serologically, including the nature of cofactors of different aPL [63], it is possible that more than one general mechanism is operating. We expect much from the use of our constructs of dimeric β_2GPI in *in vitro* and animal models. Comparison of observations made with these constructs and those of anti-β_2GPI antibodies will enable discrimination between direct effects

of dimerization and effects of Fc-receptor-mediated activation. Also, future studies in β_2GPI knock-out mice can be expected to give insights into the function of β_2GPI.

References

1. Schultze HE, Heide H, Haubt H. Über ein bisher unbekanntes niedermolekulares β2-Globulin des Humanserums. *Naturwissenschaften* 1961; 48: 719.
2. Lozier J, Takahashi N, Putnam FW. Complete amino acid sequence of human plasma beta 2-glycoprotein. *Proc Natl Acad Sci USA* 1984; 81: 3640-3644.
3. Bork P, Downing AK, Kieffer B, Campbell ID. Structure and distribution of modules in extracellular proteins. *Q Rev Biophys* 1996; 29: 119-167.
4. Hunt J, Krilis S. The fifth domain of β_2-glycoprotein I contains a phospholipid binding site (Cys281-Cys288) and a region recognized by anticardiolipin antibodies. *J Immunol* 1994; 152: 653-659.
5. Sheng YH, Sali A, Herzog H, Lahnstein J, Krilis SA. Site-directed mutagenesis of recombinant human β_2-glycoprotein I identifies a cluster of lysine residues that are critical for phospholipid binding and anti-cardiolipin antibody activity. *J Immunol* 1996; 157: 3744-3751.
6. Hunt JE, Simpson RJ, Krilis SA. Identification of a region of β_2-glycoprotein I critical for lipid binding and anti-cardiolipin antibody cofactor activity. *Proc Natl Acad Sci USA* 1993; 90: 2141-2145.
7. Horbach DA, Van Oort E, Tempelman MJ, Derksen RHWM, De Groot PhG. The prevalence of a non-phospholipid-binding form of β_2-glycoprotein I in human plasma. Consequences for the development of anti-β_2-glycoprotein I antibodies. *Thromb Haemost* 1998; 80: 791-797.
8. Mehdi H, Naqvi A, Kamboh MI. A hydrophobic sequence at position 313-316 (Leu-Ala-Phe-Trp) in the fifth domain of apolipoprotein H (β_2-glycoprotein I) is crucial for cardiolipin binding. *Eur J Biochem* 2000; 267: 1770-1776.
9. Bouma B, De Groot PhG, van den Elsen JMH, Ravelli RBG, Schouten A, Simmelink MJA, Derksen RHWM, Kroon J, Gros P. Adhesion mechanism of human β2-glycoprotein I to phospholipids based on its crystal structure. *EMBO J* 1999; 18: 5166-5174.
10. Schwartenbacher R, Zeth K, Diederichs K, Gries A, Kostner GM, Laggner P, Prassl R. Crystal structure of human β2-glycoprotein I: implication for phospholipid binding and the antiphospholipid syndrome. *EMBO J* 1999; 18: 6228-6239.
11. Lee AT, Balasubramanian K, Schroit A. β_2-glycoprotein I-dependent alterations in membrane properties. *Biochim Biophys Acta* 2000; 1509: 475-484.
12. Polz E, Kostner GM. The binding of beta 2-glycoprotein-I to human serum lipoproteins: distribution among density fractions. *FEBS Lett* 1979; 102: 183-186.
13. Bancsi LFJMM, van der Linden IK, Bertina RM. β2-glycoprotein deficiency and the risk for thrombosis. *Thromb Haemost* 1992; 67: 649-653.
14. Sheng Y, Reddel SW, Herzog H, Wang YX, Brighton T, France MP, Robertson SA, Krilis SA. Impaired thrombin generation in β_2-glycoprotein I null mice. *J Biol Chem* 2001; 276: 13817-13821.
15. Nakaya Y, Schaefer EJ, Brewer HBJr. Activation of human post heparin lipoprotein lipase by apolipoprotein H (β_2-glycoprotein I). *Biochem Biophys Res Commun* 1980; 95: 1168-1172.
16. Lin KY, Pan JP, Yang DL, Huang KT, Chang MS, Ding PYA, Chiang AN. Evidence for

inhibition of low density lipoprotein oxidation and cholesterol accumulation by apolipoprotein H (β_2-glycoprotein I). *Life Sci* 2001; 69: 707-719.
17. Schousboe I. β_2-glycoprotein I: A plasma inhibitor of the contact activation of the intrinsic blood coagulation pathway. *Blood* 1985; 66: 1086-1091.
18. Nimpf J, Wurm H, Kostner GM. Interaction of β_2-glycoprotein-I with human blood platelets: influence upon the ADP-induced aggregation. *Thromb Haemost* 1985; 54: 397-401.
19. Vallar L, Regnault V, Latger-Cannard V, Lecompte T. β_2-glycoprotein I binding to platelet microparticle membrane specificially reduces immunoreactivity of glycoproteins IIb/IIa. *Thromb Haemost* 2001; 85: 314-319.
20. Merrill JT, Zhang HW, Shen C, Butman BT, Jeffries EP, Lahita RG, Myones BL. Enhancement of protein S anticoagulant function by β_2-glycoprotein I, a major target antigen of antiphospholipid antibodies: β_2-glycoprotein I interferes with binding of protein S to its plasma inhibitor C4b-binding protein. *Thromb Haemost* 1999; 81: 748-757.
21. Roubey RAS. Immunology of the antiphospholipid antibody syndrome. *Arthritis Rheum* 1996; 39: 1444-1454.
22. Mori T, Takeya H, Nishioka J, Gabazza EC, Suzuki K. β_2-Glycoprotein I modulates the anticoagulant activity of activated protein C on the phospholipid surface. *Thromb Haemost* 1996; 75: 49-55.
23. Sheng Y, Kandiah DA, Krilis SA. Anti-β_2-glycoprotein I autoantibodies from patients with the "antiphospholipid" syndrome bind to β_2-glycoprotein I with low affinity: dimerization of β_2-glycoprotein I induces a significant increase in anti-β_2-glycoprotein I antibody affinity. *J Immunol* 1998; 161: 2038-2043.
24. Arnout J, Vermylen J. Mechanism of action of β2-glycoprotein I-dependent lupus anticoagulants. *Lupus* 1998; 7: S23-S28.
25. Chonn A, Semple SC, Cullis PR. β 2-Glycoprotein I is a major protein associated with very rapidly cleared liposomes *in vivo*, suggesting a significant role in the immune clearance of "non-self" particles. *J Biol Chem* 1995; 270: 25845-25849.
26. Manfredi AA, Rovere P, Heltai S, Galati G, Nebbia G, Tincani A, Balestrieri G, Sabbadini MG. Apoptotic cell clearance in systemic lupus erythematosus II. Role of β_2-glycoprotein I. *Arthritis Rheum* 1998; 41: 215-223.
27. Balasubramanian K, Chandra J, Schroit A. Immune clearance of phosphatidylserine-expressing cells by phagocytes. The role of β2-glycoprotein I in macrophage recognition. *J Biol Chem* 1997; 272: 31113-31117.
28. Greaves M. Antiphospholipid antibodies and thrombosis. *Lancet* 2002; 353: 1348-1353.
29. Galli M, Comfurius P, Maassen C, Hemker HC, de Baets MH, van Breda-Vriesman PJC, Barbui T, Zwaal RFA, Bevers EM. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein factor. *Lancet* 1990; 335: 1544-1547.
30. McNeil HP, Simpson RJ, Chesterman CN, Krilis SA. Antiphospholipid antibodies are directed against a complex antigen that includes a lipid binding inhibitor of coagulation: β_2-glycoprotein I (apolipoprotein H). *Proc Natl Acad Sci USA* 1990; 87: 4120-4124.
31. Matsuura E, Igarashi Y, Fujimoto M, Ichikawa K, Koike T. Anticardiolipin cofactor(s)

and differential diagnosis of autoimmune disease. *Lancet* 1990; 336: 177-178.
32. Oosting JD, Derksen RHWM, Entjes HTI, Bouma BN, De Groot PhG. Lupus anticoagulant activity is frequently dependent on the presence of β_2-glycoprotein I. *Thromb Haemost* 1992; 67: 499-502.
33. Gharavi AE, Sammaritano LR, Wen J, Elkon KB. Induction of antiphospholipid antibodies by immunization with β2-glycoprotein I (Apolipoprotein H). *J Clin Invest* 1992; 90: 1105-1109.
34. Blank M, Faden D, Tincani A, Kopolovic J, Goldberg I, Gilburd B, Allegri F, Balestrieri G, Valesini G, Shoenfeld Y. Immunization with anticardiolipin cofactor (beta-2-glycoprotein I) induces experimental antiphospholipid syndrome in naive mice. *J Autoimmun* 1994; 7: 441-455.
35. Pierangeli SS, Liu XW, Anderson G, Barker JH, Harris EN. Thrombogenic properties of murine anti-cardiolipin antibodies induced by $\beta_2$glycoprotein 1 and human immunoglobulin G antiphospholipid antibodies. *Circulation* 1996; 94: 1746-1751.
36. Iverson GM, Victoria EJ, Marquis DM. Anti-β2 glycoprotein I (β2GPI) autoantibodies recognize an epitope on the first domain of β2GPI. *Proc Natl Acad Sci USA* 1998; 95: 15542-15546.
37. McNeeley PA, Dlott JS, Furie RA, Jack RM, Ortel TL, Triplett DA, Victoria EJ, Linnik MD. β_2-glycoprotein I-dependent anticardiolipin antibodies preferentially bind the amino terminal domain of β_2-glycoprotein I. *Thromb Haemost* 2001; 86: 595.
38. Cheng-De Y, Shun-Le C, Nan S, Ming Q, Feng X. Detection of anti-recombinant β_2-glycoprotein I and anti-recombinant β_2-glycoprotein I fifth-domain antibodies in sera from patients with systemic lupus erythematosus. *Rheumatol Int* 1998; 18: 5-10.
39. George J, Gilburd B, Hojnik M, Levy Y, Langevitz P, Matsuura E, Koike T, Shoenfeld Y. Target recognition of β_2-glycoprotein I (β_2GPI)-dependent anticardiolipin antibodies: Evidence for involvement of the fourth domain of β_2GPI in antibody binding. *J Immunol* 1998; 160: 3917-3923.
40. Takeya H, Mori T, Gabazza EC, Kuroda K, Deguchi H, Matsuura E, Ichikawa K, Koike T, Suzuki K. Anti-β2-glycoprotein I (β2GPI) monoclonal antibodies with lupus anticoagulant-like activity enhance the β2GPI binding to phospholipids. *J Clin Invest* 1997; 99: 2260-2268.
41. Blank M, Shoenfeld Y, Cabilly S, Heldman Y, Fridkin M, Katchalski-Kater E. Prevention of experimental antiphospholipid syndrome and endothelial cell anctivation by synthetic peptides. *Proc Natl Acad Sci USA* 1999; 96: 5168.
42. Blank M, Krause I, Fridkin M, Keller N, Kopolovic J, Goldberg I, Tobar A, Shoenfeld Y. Bacterial induction of autoantibodies to β2-glycoprotein-I accounts for the infectious ethiology of the antiphospholipid syndrome. *J Clin Invest* 2002; 109: 797-804.
43. Gharavi AE, Pierangeli SS, Espinola RG, Liu X, Colden-Stanfield M, Harris EN. Antiphospholipid antibodies induced in mice by immunization with a cytomegalovirus-derived peptide cause thrombosis and activation of endothelial cells in vivo. *Arthritis Rheum* 2002; 46: 545-552.
44. Meroni PL, Raschi E, Camera M, Testoni C, Nicoletti F, Tincani A, Khamashta MA, Balestrieri G, Tremoli A, Hess DC. Endothelial activation by aPL: A potential pathogenic mechanism for clinical manifestations of the syndrome. *J Autoimmun* 2000; 15: 237-

240.
45. Carreras LO, Machin SJ, Deman R, Defreyn G, Vermylen J, Spitz B, Van Assche A. Arterial thrombosis, intrauterine death and "lupus" anticoagulant: detection of immunoglobulin interfering with prostacyclin formation. *Lancet* 1981; 1: 244-246.
46. Carreras LO, Forastiero R, Martinuzzo M. Interaction between antiphospholipid antibodies and eicosanoids. In: Khamashta MA, ed. *The Hughes Syndrome.* London: Springer-Verlag; 2000:337-347.
47. Hasselaar P, Derksen RH, Blokzijl L, De Groot PG. Thrombosis associated with antiphospholipid antibodies cannot be explained by effects on endothelial and platelet prostanoid synthesis. *Thromb Haemost* 1988; 59: 80-85.
48. Forastiero RR, Martinuzzo M, Carreras LO, Maclouf J. Anti-β_2-glycoprotein I antibodies and platelet activation in patients with antiphospholipid syndrome: association with increased excretion of platelet-derived thromboxane urinary metabolites. *Thromb Haemost* 1998; 79: 42-45.
49. Martinuzzo M, Maclouf J, Carreras LO, Lé vy-Toledano S. Antiphospholipid antibodies enhance thrombin-induced platelet activation and thromboxane formation. *Thromb Haemost* 1993; 70: 667-671.
50. Escolar G, Reverter JC, Lopez-Soto A, Garrido M, Cervera R, Ingelmo M, Castillo R, Ordinas A. Plasma from systemic lupus erythematosus patient with antiphospholipid antibodies promotes platelet aggregation. *Arterioscler Thromb* 1992; 12: 196-200.
51. Lin Y-L, Wang C-T. Activation of human platelets by the rabbit anticardiolipin antibodies. *Blood* 1992; 80: 3135-3143.
52. Arvieux J, Roussel B, Pouzol P, Colomb MG. Platelet activating properties of murine monoclonal antibodies to β_2-glycoprotein I. *Thromb Haemost* 1993; 70: 336-341.
53. Dunoyer-Geidre S, Kruithof EKO, Galve-de Rochemonteix B, Rosnoblet C, Gruenberg J, Reber G, de Moerloose P. Localization of β_2-glycoprotein I in late endosomes of human endothelial cells. *Thromb Haemost* 2001; 85: 903-907.
54. Arnout J, Wittevrongel C, Vanrusselt M, Hoylaerts M, Vermylen J. Beta-2-glycoprotein I dependent lupus anticoagulants form stable bivalent antibody beta-2-glycoprotein I complexes on phospholipid surfaces. *Thromb Haemost* 1998; 79: 79-86.
55. Willems GM, Janssen MP, Pelsers MAL, Comfurius P, Galli M, Zwaal RFA, Bevers EM. Role of divalency in the high-affinity binding of anticardiolipin antibody-β_2-glycoprotein I complexes to lipid membranes. *Biochemistry* 1996; 35: 13833-13842.
56. Arnout J. Antiphospholipid syndrome: diagnostic aspects of lupus anticoagulants. *Thromb Haemost* 2001; 86: 83-91.
57. Regnault V, de Maistre E, Wahl D, Lecompte T. Monovalent binding of autoantibodies to β_2-glycoprotein I, detected using surface plasmon resonance at low antigen density. *Br J Haematol* 2000; 109: 187-194.
58. Lutters BCH, Meijers JCM, Derksen RHWM, Arnout J, De Groot PG. Dimers of β_2-glycoprotein I mimic the in vitro effects of β_2-glycoprotein I-anti-β_2-glycoprotein I antibody complexes. *J Biol Chem* 2001; 276: 3060-3067.
59. Ma K, Simantov R, Zhang J-C, Silverstein R, Hajjar KA, McCrae KR. High affinity binding of β_2-glycoprotein I to human endothelial cells is mediated by annexin II. *J Biol Chem* 2000; 275: 15541-15548.

60. Moestrup SK, Schousboe I, Jacobsen C, Leheste JR, Christensen EI, Willnow TE. β_2-glycoprotein I (apolipoprotein H) and β_2-glycoprotein I-phospholipid complex harbor a recognition site for the endocytic receptor megalin. *J Clin Invest* 1998; 102: 902-909.
61. Arnout J. The pathogenesis of the antiphospholipid syndrome: a hypothesis based on parallelism with heparin-induced thrombocytopenia. *Thromb Haemost* 1996; 75: 536-541.
62. Jankowski M, Vreys I, Wittevrongel C, Boon D, Vermylen J, Hoylaerts MF, Arnout J. Thrombogenecity of beta2-glycoprotein I dependent antiphospholipid antibodies in a photochemically induced thrombosis model in the hamster. *Blood* 2002 [epub ahead of print].
63. Rand JH. Molecular pathogenesis of the antiphospholipid syndrome. *Circ Res* 2002; 90: 29-37.

Chapter 3

Dimers of β_2-glycoprotein I mimic the in vitro effects of β_2-glycoprotein I-anti-β_2-glycoprotein I antibody complexes

Bianca C.H. Lutters[1,3], Joost C.M. Meijers[4], Ronald H.W.M. Derksen[2], Jef Arnout[5], Philip G. de Groot[1,3]

Departments of [1]Haematology and [2]Rheumatology and Clinical Immunology, University Medical Center, Utrecht and [3]Institute of Biomembranes, Utrecht University, [4]Department of Vascular Medicine, Academic Medical Center, Amsterdam, The Netherlands, and the [5]Center for Molecular and Vascular Biology, University of Leuven, Leuven, Belgium.

Journal of Biological Chemistry 276, 3060-3067, 2001

Abstract

Anti-β_2-glycoprotein I antibodies are thought to cause lupus anticoagulant activity (LAC) by forming bivalent complexes with β_2-glycoprotein I (β_2GPI). In order to test this hypothesis, chimeric fusion proteins were constructed of the dimerization domain (apple4) of factor XI and β_2GPI. Both a covalent (apple4-β_2GPI) and a non-covalent (apple4-C321S-β_2GPI) chimer were constructed. As controls, apple2-β_2GPI and apple4-C321S-β_2GPI-W316S, in which β_2GPI-W316S is not able to bind to phospholipids, were made. In a phospholipid binding assay, apple4-β_2GPI and apple4-C321S-β_2GPI were able to bind to phospholipids with a 35 times higher affinity than plasma-derived β_2GPI and apple2-β_2GPI. Apple4-C321S-β_2GPI-W316S did not bind at all. Only apple4-β_2GPI and apple4-C321S-β_2GPI were able to bind to adhered platelets as shown by immunofluorescence. Using the prothrombin time, which was the most responsive coagulation assay, the clotting time was approximately doubled when 200 µg/ml of apple4-β_2GPI or apple4-C321S-β_2GPI was added. Addition of 200 µg/ml plasma-derived β_2GPI, apple2-β_2GPI or apple4-C321S-β_2GPI-W316S did not affect clotting time. Clotting time could be corrected with the addition of extra phospholipids, which is indicative for lupus anticoagulant activity. Extra prolongation of clotting time for apple4-β_2GPI and apple4-C321S-β_2GPI was achieved by the addition of monoclonal antibodies against β_2GPI. In conclusion, dimerization of β_2GPI explains the *in vitro* observed effects of β_2GPI-anti-β_2GPI antibody complexes.

Introduction

β_2-glycoprotein I (β_2GPI), also known as apolipoprotein H, is a single chain protein present in plasma at a concentration of approximately 200 µg/ml. It consists of 326 amino acids and has a molecular mass of 42 kDa. β_2GPI is a member of the short consensus repeat (SCR) or complement control protein (CCP) superfamily. The first four domains of β_2GPI consist of ~ 60 amino acids and contain 2 conserved disulfide bonds. The fifth domain, comprising 84 amino acids, is different with an extra C-terminal tail, resulting in a C-terminal loop and an extra disulfide bond [1-5]. This domain is important for phospholipid binding as has been shown by several studies. Experiments using mutated forms of β_2GPI and peptides spanning the fifth domain of β_2GPI, indicated that residues Cys281 to Cys288 are important for phospholipid binding [6-8]. Furthermore, analysis of a naturally occurring polymorphism, in which Trp316 is mutated to a serine [9] showed that Trp316 is essential for phospholipid binding. Mehdi *et al.* showed that a hydrophobic sequence at position 313-316 in the fifth domain is crucial for cardiolipin binding [10]. The recent elucidation of the crystal structure of β_2GPI [11] showed that a patch of 14 positively charged amino acids in the fifth domain is important for electrostatic interaction with negatively charged phospholipids. It was also shown that the amino acids at positions 311-317 form a membrane-insertion loop, which yields specificity for lipid bilayers. At present, the *in vivo* function of β_2GPI is unknown. *In vitro*, it has been shown to influence coagulation (both procoagulant and anticoagulant)[12-19], platelet function [20-23], lipoprotein metabolism [24-27], and apoptosis [24].

The persistent presence of antiphospholipid antibodies in plasma is a risk factor for thrombosis, pregnancy loss and thrombocytopenia. These clinical and serological observations together are termed the antiphospholipid syndrome [28-33]. Initially it was thought that the antiphospholipid antibodies were directed against phospholipids directly, but now it is generally accepted that these antibodies are directed against phospholipid-bound proteins, such as β_2GPI [34-36] and prothrombin [37]. Antiphospholipid antibodies are a very heterogeneous group of antibodies, which can be subdivided into lupus anticoagulants (LAC) and anticardiolipin antibodies (aCL), of which the latter can bind to β_2GPI immobilized on cardiolipin surfaces [38,39]. Antibodies with LAC activity are detected by their ability to prolong phospholipid dependent coagulation assays [40,41]. The prolongation can not be corrected by the addition of normal pool plasma, but can be corrected by the addition of extra phospholipids. Some of these lupus anticoagulants depend on prothrombin to prolong clotting time *in vitro* [42], but most of them depend on β_2GPI [43,44].

Two possible explanations for the mechanism of action of β_2GPI-anti-β_2GPI antibody complexes are suggested. First, β_2GPI undergoes a conformational change when it interacts with anti-β_2GPI antibodies [45-50]. Second, β_2GPI is able to form bivalent complexes when it interacts with an anti-β_2GPI antibody [51-53]. The final result is a strongly increased affinity of β_2GPI for negatively charged phospholipids. Due to the increased affinity, β_2GPI is then able to compete with clotting factors for the phospholipid surface, resulting in the *in vitro* prolongation of coagulation [54].

Here, a covalent and a non-covalent chimeric dimer of β_2GPI are constructed to determine the role of bivalent β_2GPI complexes without the intervention of antibodies. It is shown that chimeric dimers of the dimerization domain of coagulation factor XI (apple4)[55,56]

and β_2GPI are able to induce a strongly increased binding to phospholipids resulting in a LAC activity.

Materials and Methods

Construction of expression vectors

Human β_2GPI cDNA, kindly provided by Dr. T. Kristensen (University of Aarhus, Aarhus, Denmark) was subcloned into the BamHI site of pUC18. The non-phospholipid binding form β_2GPI-W316S [9] was constructed by site-directed mutagenesis (Quickchange site-directed mutagenesis kit, Stratagene, La Jolla, CA, USA) of pUC18-β_2GPI with the primers β_2GPI-f (CA GTT CTC *TGG* CTT TTT GCA AAA CTG ATG CAT CCG ATG) and β_2GPI-r (CAT CGG ATG CAT CAG TTT TCG AAA AAG *CCA* GAG AAC TG). The sequences in italic indicate the mutated codon. The sequence encoding the mature β_2GPI protein was amplified with the primers β_2GPI-XhoI (C CCT CGA GGA CGG ACC TGT CCC AAG CC) and β_2GPI-XbaI (GC TCT AGA AAA CAA GTG TGA CAT TTT ATG TGG A) in a PCR reaction. To construct chimeric fusion proteins of the dimerization domain of factor XI (apple4) and β_2GPI the PCR product was cloned with *XhoI* and *XbaI* (underlined in β_2GPI-*XhoI* and β_2GPI-*XbaI*, respectively) into the vectors apple4-tissue-type plasminogen activator (tPA)-S478A and apple4-C321S-tPA-S478A[56]. In this way apple4-β_2GPI and apple4-C321S-β_2GPI were constructed. As controls, the chimers apple2-β_2GPI and apple4-C321S-β_2GPI-W316, in which β_2GPI-W316S is not able to bind to phospholipids, were made. For apple2-β_2GPI, the PCR product was cloned into apple2-tPA-S478A with *XhoI* and *XbaI* [56]. For apple4-C321S-β_2GPI-W316S the PCR product was cloned into apple4-C321S-tPA-S478A [56]. Sequence analysis was performed to confirm correct amplification of the β_2GPI cDNA.

Cell culture, transfection, expression and purification of fusion proteins

Transfection of baby hamster kidney cells was performed as described previously [55]. Expression of all fusion constructs was performed in conditioned serum-free medium (DMEM F12 supplemented with 1% UltroserG; Life Technologies, Inc., Paisley, U.K.). Protein expression was measured using a total β_2GPI-ELISA as described before [9]. Apple-β_2GPI fusion proteins were purified using a monoclonal antibody to β_2GPI (21B2) bound to CNBr-activated Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden)[9]. Bound fusion proteins were eluted with 0.1 M glycine (pH 2.7). The purified proteins were immediately neutralized with 1 M Tris, pH 9. Apple4-β_2GPI, apple4-C321S-β_2GPI and apple4-C321S-β_2GPI-W316S containing fractions were dialyzed against 50 mM Na_2HPO_4, pH 7.0, containing 50 mM NaCl and were subjected to further purification on a mono S column using FPLC (Amersham Pharmacia Biotech, Uppsala, Sweden). Fusion proteins were eluted with a linear salt gradient from 50 mM NaCl to 1 M NaCl. All chimers were dialyzed against tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4). Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) according to the instructions of the manufacturer, and with bovine serum albumin (BSA) as a standard. The yield varied from 5 to 10 µg protein per ml culture medium. Purified constructs were analyzed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of proteins

β_2GPI was isolated from freshly frozen citrated human plasma as described previously [33]. In short, dialyzed human plasma was applied to a DEAE-Sephadex A50 column, subsequently followed by protein G-Sepharose, S-Sepharose and finally heparin-Sepharose chromatography (all Sepharoses were obtained from Amersham Pharmacia Biotech, Uppsala, Sweden). Bound proteins were eluted with a linear salt gradient. Afterwards, β_2GPI was dialyzed against TBS. Purity of the protein was checked with SDS-PAGE analysis. Fab fragments of the monoclonal anti-β_2GPI-antibody 23H9, which is directed against domain 2, were generated using an ImmunoPure Fab Kit according to the instructions of the manufacturer (Pierce, Rockford IL, USA) . In short, IgG was incubated with papain-coated beads overnight at 37°C and Fc fragments and uncut IgG were removed on a protein-A sepharose column. After SDS-PAGE 23H9 Fab appeared as one band with a molecular weight of 25 kDa under reducing conditions. Concentration of the proteins was determined using a BCA protein assay.

Gel filtration studies

Apple-β_2GPI fusion proteins and β_2GPI were applied to Superdex 200 gel filtration column using FPLC equipment. The column was equilibrated with TBS, pH 7.4, at a flow rate of 0.5 ml/min. The absorbance of the eluent was monitored at 280 nm. Molecular weights were determined by comparison to a standard curve of chymotrypsinogen A (Mr 25 kDa), ovalbumin (43 kDa), BSA (67 kDa), aldolase (158 kDa) and thyroglobulin (669 kDa).

Preparation of phospholipid vesicles

Phospholipid vesicles containing 20% phosphatidylserine (PS)/40% phosphatidylcholine (PC)/40% phosphatidylethanolamine (PE) or 20% PS/80% PC (Sigma, St. Louis, MO, USA) were prepared according to Brunner *et al.* [57] with some modifications as described by van Wijnen *et al.* [58]. The phospholipid content of the fractions was determined by phosphate analysis [59].

Binding of apple-β_2GPI fusion proteins to phospholipids

To measure binding of apple-β_2GPI chimers and β_2GPI to phospholipids a solid phase binding assay according to Horbach *et al.* [9] with some modifications was used. In short, 25 µM of phospholipids (20% PS/80% PC and 20% PC/40% PC/40% PE vesicles) were coated in 96-well ELISA plates (Costar, Cambridge, MA, USA) overnight at 4°C. Wells were blocked with TBS/0.5% gelatin for 2 hours at 37°C, followed by successive incubations with apple-β_2GPI chimers or β_2GPI (50 µL/well) for 1.5 hours at 37 °C, and monoclonal antibody 2B2 (3 µg/mL; 50 µL/well; 1.5 hours at 37°C). Afterwards, wells were incubated with a rabbit-anti-mouse peroxidase-conjugated antibody (1:1000 diluted; 50 µL/well; Dako, Glostrup, Denmark) and developed using o-phenylenediamine. All samples were diluted in TBS/0.5% gelatin and after each incubation step, wells were washed three times with TBS. Non-specific binding was determined in wells where phospholipids were absent.

Spraying of coverslips

Glass coverslips (18 x 18 mm; Menzel Glä ser, Braunschweig, Germany) were soaked overnight in chromosulfuric acid, rinsed thoroughly with deionized water, and air-dried. Human placenta collagen type III (Sigma, St Louis, MO, USA) was solubilized in 50 mM acetic acid (1 mg/mL) and sprayed on glass coverslips at a final density of 30 µg/cm^2 with a retouching air-brush (Badger model 100; Badger Brush Co, Franklin Park, IL, USA).

Perfusions

Perfusions were performed in a single pass perfusion chamber. The perfusions were performed under non-pulsatile flow conditions using a parallel plate perfusion with a slit height of 0.1 mm and a slit width of 2 mm. Experiments were done with a flow rate of 58 µL/min (shear rate 300 s^{-1})[60] and a perfusion time of 3 min with collagen type III as a surface. Prewarmed (5 min 37°C) citrated blood (1/10 vol 3.2% trisodium citrate) was drawn through the perfusion chamber by a Harvard infusion pump (pump 22, model 2400-004; Natick, MA, USA). Afterwards, the coverslips were removed and rinsed with 10 mM HEPES buffer, containing 150 mM NaCl, followed by fixation with 3% paraformaldehyde/0.005% glutardialdehyde in phosphate-buffered saline (PBS), pH 7.4 for 30 min at room temperature. Fixed glass coverslips were stored till use in PBS/1% paraformaldehyde/0.002% glutardialdehyde, pH 7.4.

Immunofluorescence studies

Coverslips were washed three times with PBS and blocked for 10 min with PBS/1% BSA/0.1% glycine, pH 7.4. After this, the coverslips were incubated for 1 h at 37°C with apple-β_2GPI fusion proteins (10 µg/mL) diluted in PBS/1% BSA/0.1 % glycine, pH 7.4, followed by three times washing with PBS and 10 min blocking with PBS/1% BSA/0.1% glycine, pH 7.4 at room temperature. Then the coverslips were incubated for 45 min with the monoclonal anti-β_2GPI antibody diluted in PBS/1% BSA/0.1% glycine, pH 7.4 (10 µg/mL) at 37°C, followed by three times washing with PBS and 10 min blocking with PBS/1% BSA/0.1% glycine, pH 7.4 at room temperature. Afterwards, the coverslips were incubated with a fluorescein isothiocyanate (FITC)-labeled goat-anti-mouse antibody (Becton Dickinson, San Jose, CA, USA), diluted 1:20 in PBS/1% BSA/0.1% glycine, pH 7.4 for 45 min at 37°C, followed by three times washing with PBS. Lastly, the coverslips were mounted in MOWIOL 4-88 (Calbiochem, La Jolla, CA, USA) and 0.1% PPD (para-phenylene diamine; Sigma, St Louis, MO, USA).

β_2GPI-deficient plasma

β_2GPI-deficient plasma was made using a monoclonal antibody to β_2GPI (21B2) coupled to CNBr-activated Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden)[9]. Pooled normal plasma (10 mL) was applied to the column and the flow through plasma was collected in fractions of 0.5 ml and directly frozen in liquid nitrogen and stored at -70°C. Levels of β_2GPI were determined using a total-β_2GPI ELISA as described previously [9] and fractions containing less than 10% β_2GPI were considered deficient.

Coagulation assays

All assays were performed in a KC-10 coagulometer (Amelung, Lemgo, Germany). For prothrombin time (PT), 25 µL of pooled normal plasma and 25 µL of apple-β_2GPI fusion

protein or plasma-derived β_2GPI (final concentration 0-200 µg/mL) were incubated for 30 min at 4°C followed by an incubation of 1.5 min at 37°C. Clotting was initiated by the addition of 50 µL of Innovin (Dade Behring, Marburg, Germany).

Activated partial thromboplastin time (aPTT) was measured with a test which has been sensitized to aid the detection of lupus anticoagulants, the PTT-LA (Diagnostica Stago, Asnieres-sur-Seine, France). 25 µL of pooled normal plasma and 25 µL of apple-β_2GPI fusion protein or plasma-derived β_2GPI (final concentration 0-200 µg/mL) were incubated for 30 min at 4°C. After addition of 50 µL of PTT-LA reagent, the samples were incubated for 3 min at 37°C. Clotting was initiated by the addition of 50 µL of 25 mM $CaCl_2$.

Dilute Russell's viper venom time (dRVVT) was measured with LA-screen (Gradipore LTD, North Ryde, Australia). 25 µL of pooled normal plasma and 25 µL of apple-β_2GPI fusion protein or plasma-derived β_2GPI (final concentration 0-200 µg/mL) were incubated for 30 min at 4°C, followed by an incubation of 1.5 min at 37°C. Clotting was initiated by addition of 50 µL of LA-screen.

Correction of clotting time was performed using PT and extra addition of cephalin. 25 µL of pooled normal plasma and 25 µL of a mixture of apple-β_2GPI fusion protein (final concentration 0-200 µg/mL) and cephalin (final concentration was 3.2 times the concentration used in an aPTT according to the instructions of the manufacturer; aPTT reagent, Boehringer Mannheim, Mannheim, Germany) were incubated for 30 min at 4°C followed by an incubation of 1.5 min at 37°C. Clotting was initiated by the addition of 50 µL of Innovin.

The effect of monoclonal antibodies against β_2GPI on clotting time in the presence of plasma-derived β_2GPI or apple-β_2GPI dimeric chimers was determined using PT. Two monoclonal anti-β_2GPI antibodies with LAC activity were used. One was directed against domain four (19H9) and one was directed against domain two (23H9) of β_2GPI [52]. 25 µL of β_2GPI-deficient plasma and 25 µL of a mixture of plasma-derived β_2GPI or apple-β_2GPI fusion protein (final concentration 0 or 200 µg/mL) and a monoclonal antibody against β_2GPI (final concentration 100 µg/mL) or the Fab fragment of the monoclonal antibody 23H9 (final concentration 100 µg/mL were incubated for 30 min at 4°C, followed by an incubation of 1.5 min at 37°C. Clotting was initiated by the addition of 50 µLof Innovin (Dade Behring, Marburg, Germany).

Results were indicated in clotting time (s; mean ± SD, n = 3). Clotting times ≥ mean clotting time + 20% we considered being positive. For the coagulation assays using monoclonal antibodies against β_2GPI only one representative experiment was shown.

Results

Expression and purification of fusion proteins

To study the effect of dimeric β_2GPI on phospholipid binding and clotting time, chimeric constructs of β_2GPI fused to the C-terminus of the dimerization domain, apple4, of factor XI were made (fig. 1A). Both a covalent (apple4-β_2GPI) and a non-covalent (apple4-C321S-β_2GPI) chimeric dimer were made. As controls, apple2-β_2GPI and apple4-C321S-β_2GPI-W316S, in which β_2GPI-W316S is not able to bind to phospholipids, were constructed. Baby hamster kidney cells were transfected with expression vectors containing chimeric apple-

β_2GPI constructs. Protein expression was confirmed with a total β_2GPI-ELISA. The proteins were affinity purified with a monoclonal antibody against β_2GPI. Apple4-β_2GPI, apple4-C321S-β_2GPI and apple4-C321S-β_2GPI-W316S were further purified by FPLC with a mono S column. After purification, the fusion proteins were applied to 10% SDS-PAGE under reducing and nonreducing conditions and stained by Coommassie Brilliant Blue (fig.1B and C). Under nonreducing conditions apple4-C321S-β_2GPI and apple4-C321S-β_2GPI-W316S migrated as monomers with an apparent molecular weight of 47 kDa. Apple2-β_2GPI migrated slightly

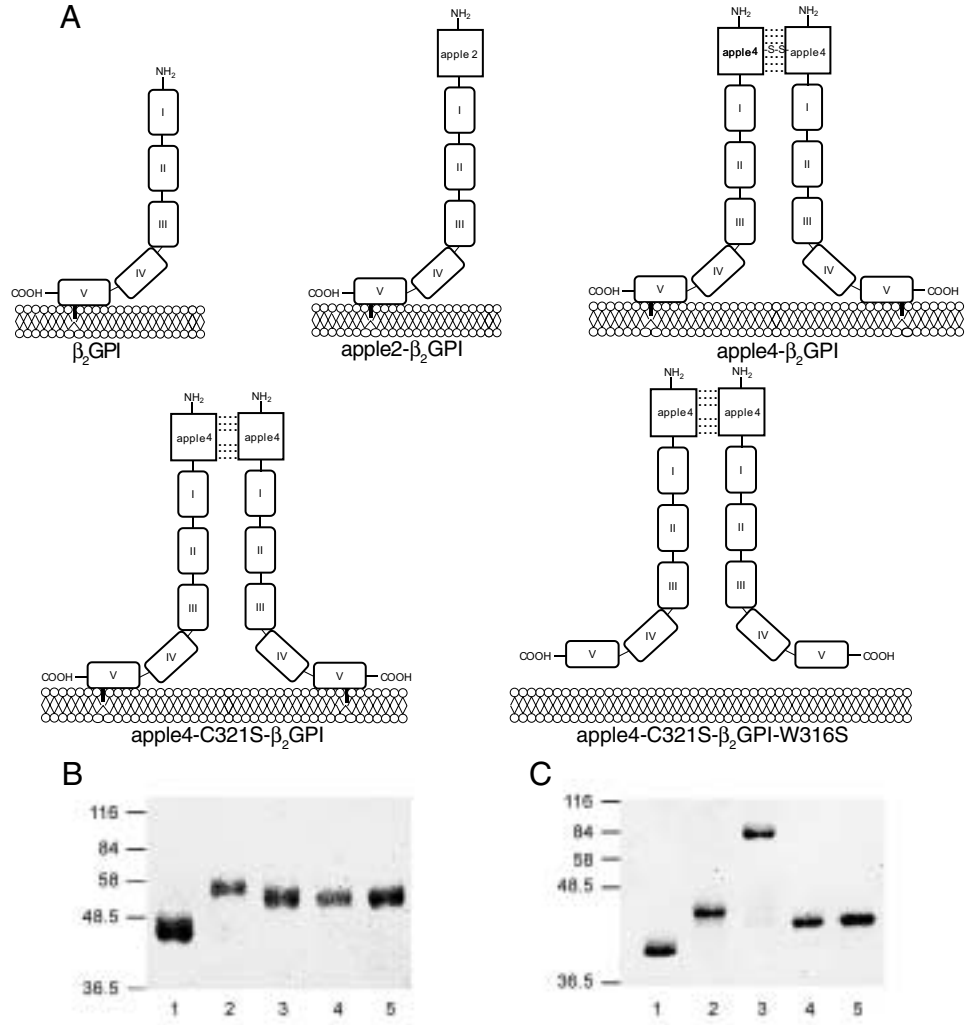

FIG. 1. **SDS-PAGE analysis of apple-β_2GPI fusion proteins.** *A*, schematic representation of used proteins. Purified plasma-derived β_2GPI (lanes 1), apple2-β_2GPI (lanes 2), apple4-β_2GPI (lanes 3), apple4-C321S-β_2GPI (lanes 4), and apple4-C321S-β_2GPI-W316S (lanes 5) were analyzed on 10% polyacrylamide gels under reducing (*B*) and nonreducing (*C*) conditions. The proteins were visualized by Coommassie staining. The molecular masses of prestained markers are indicated in kilodaltons.

slower than apple4-C321S-β_2GPI and apple4-C321S-β_2GPI-W316S at 49 kDa, which is probably caused by glycosylation of apple2. Apple4-β_2GPI migrated as a dimer under nonreducing conditions with a molecular weight of approximately 110 kDa. Plasma-derived β_2GPI migrated with a molecular weight of 42 kDa under nonreducing conditions. Upon reduction, all fusion proteins migrated as monomers with a molecular weight of 59 kDa for apple2-β_2GPI and 57 kDa for the other fusion proteins. Plasma-derived β_2GPI migrated with a molecular weight of 49 kDa under reducing conditions. Western blot analysis with polyclonal anti-β_2GPI and anti-factor XI antibodies showed reactivity with all apple-β_2GPI fusion proteins (results not shown).

Gel filtration studies

To determine whether apple4-C321S-β_2GPI was dimeric in the native state, gel filtration studies were performed. Apple4-β_2GPI eluted with a molecular weight of 115 kDa, indicating that it was a dimer, as was also shown by SDS-PAGE analysis. Apple4-C321S-β_2GPI eluted with molecular weight of 115 kDa demonstrating that apple4-C321S-β_2GPI was also a dimer under a non-denaturating condition. Apple2-β_2GPI eluted with a molecular weight of 61 kDa, indicating that it was indeed a monomer. These results were consistent with the findings of Meijers *et al.*[56] for chimeric apple-tPA constructs. Plasma-derived β_2GPI eluted with a molecular weight of 49 kDa, which is consistent with its known molecular weight.

Binding of apple-β_2GPI chimers to immobilized phospholipids

The phospholipid binding features of apple-β_2GPI fusion proteins were tested in a solid phase binding assay. Phospholipid vesicles (25 µM, 20% PS/80% PC or 20% PS/40% PC/40% PE) were immobilized on 96-well ELISA plates and binding of plasma-derived β_2GPI and apple-β_2GPI chimers was measured. As shown in fig. 2A, half maximal binding of apple4-β_2GPI and apple4-C321S-β_2GPI to phospholipid vesicles (20% PS/80% PC) occurred at concentrations as low as 0.9 and 3.7 µg/mL, respectively. For all proteins binding of apple-β_2GPI fusion proteins to 20% PS/40% PC/40% PE vesicles (fig. 2B) was stronger than binding to 20% PS/80% PC vesicles (fig. 2A). Half maximal binding of apple4-β_2GPI and apple4-C321S-β_2GPI to 20% PS/40% PC/40% PE vesicles was observed at concentrations of 0.3 µg/mL and 1.4 µg/mL, respectively. Binding of apple4-C321S-β_2GPI at concentrations of 0.5-16 µg/mL was lower than binding of apple4-β_2GPI at these concentrations for both 20% PS/80% PC and 20% PS/40% PC/40% PE vesicles. For both vesicle types, plasma-derived β_2GPI and apple2-β_2GPI only showed little binding to phospholipid vesicles at a concentration of 32 µg/mL. Apple4-C321S-β_2GPI-W316S was not able to bind to phospholipid vesicles (both 20% PS/80% PC and 20% PS/40% PC/40% PE vesicles) at concentrations as high as 32 µg/mL, which was expected, since β_2GPI-W316S is not able to bind to phospholipid vesicles [9]. To determine if plasma-derived β_2GPI and apple2-β_2GPI were able to bind to phospholipids (20% PS/80% PC vesicles) at higher concentrations, concentrations to 100 µg/mL were used. As shown in figure 2C, plasma-derived β_2GPI and apple2-β_2GPI were able to bind to phospholipids at a concentration of 100 µg/mL. Half maximal binding of plasma-derived β_2GPI occurred at 32 µg/mL (results not shown). Even at a concentration of 100 µg/mL, apple4-C321S-β_2GPI-W316S was unable to bind to immobilized phospholipids (fig. 2C). These results demonstrated that apple4-β_2GPI and apple4-C321S-β_2GPI were able to bind to phospholipids with increased affinity compared to plasma-derived β_2GPI and apple2-β_2GPI.

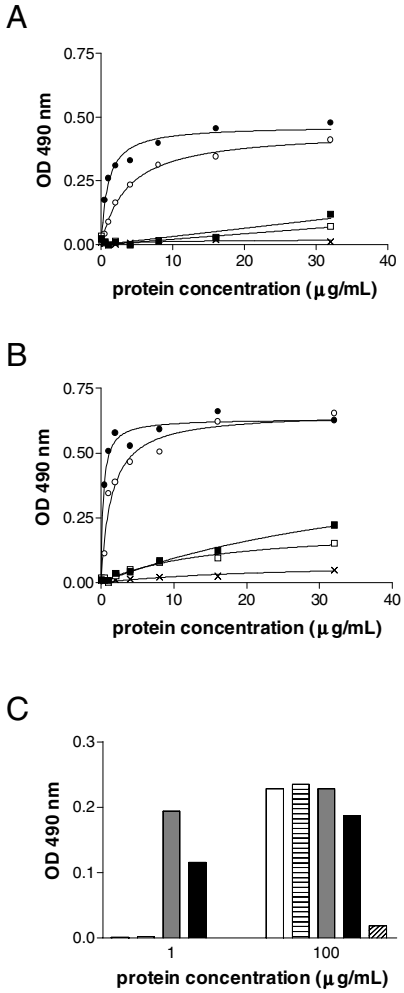

FIG. 2. **Binding of apple-β_2GPI chimers to immobilized phospholipids.** 20% PS/80% PC vesicles (*A and C*) or 20% PS/40% PC/40% PE vesicles (*B*) were coated on 96-well ELISA plates. *A and B*, increasing concentrations (0-32 µg/ml) of purified plasma-derived β_2GPI (black squares), apple2-β_2GPI (open squares), apple4-β_2GPI (black dots), apple4-C321S-β_2GPI (open dots), and apple4-C321S-β_2GPI-W316S (crosses) were assayed for phospholipid binding. *C,* binding of 1 and 100 µg/mL of plasma-derived β_2GPI (white bar), apple2-β_2GPI (lined bar), apple4-β_2GPI (grey bar), apple4-C321S-β_2GPI (black bar), and apple4-C321S-β_2GPI-W316S (shaded bar) to phospholipids.

Binding of apple-β_2GPI fusion proteins to platelets

Platelets expose phosphatidylserine immediately after adhesion to collagen [61]. To test the binding of apple-β_2GPI chimers to physiological phospholipid membranes, perfusion experiments were performed. Whole blood was perfused over collagen type III-coated glass coverslips at a shear rate of 300 s^{-1} to allow platelets to bind to collagen. After fixation of the adhered platelets, plasma-derived β_2GPI or apple-β_2GPI fusion proteins (10 µg/mL) were allowed to bind to the platelets. Binding of fusion proteins was determined by immunofluorescence. As shown in fig. 3, apple4-β_2GPI and apple4-C321S-β_2GPI were able to bind to the platelets, while plasma-derived β_2GPI, apple2-β_2GPI and apple4-C321S-β_2GPI-W316S were unable to bind.

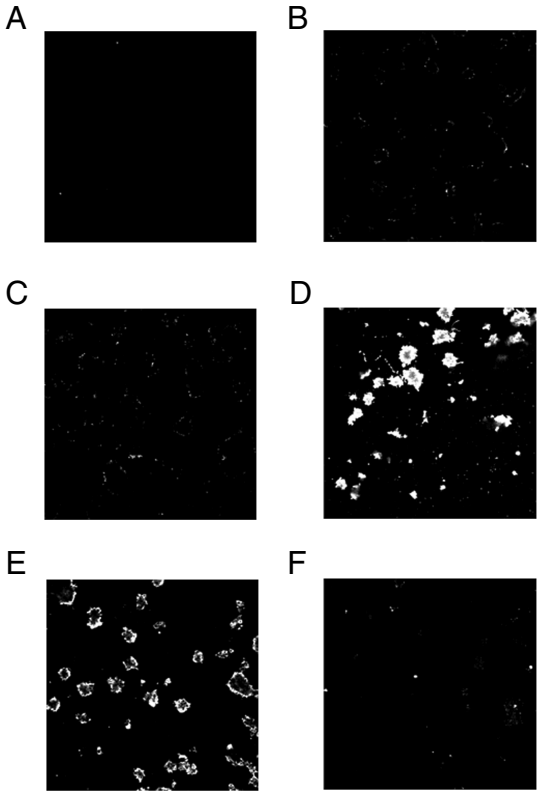

FIG. 3. **Binding of apple-β_2GPI chimers to platelets adhered to collagen type III.** No protein (*A*), plasma-derived β_2GPI (*B*), apple2-β_2GPI (*C*), apple4-β_2GPI (*D*), apple4-C321S-β_2GPI (*E*) or apple4-C321S-β_2GPI-W316S (*F*) at a concentration of 10 µg/mL was bound to adhered platelets. Binding was determined using immunofluorescence.

Effect of apple-β_2GPI fusion proteins on in vitro coagulation tests

To study the effect of apple-β_2GPI chimers on coagulation, several coagulation assays were performed. Increasing concentrations of plasma-derived β_2GPI and apple-β_2GPI fusion proteins (50-200 µg/mL), diluted in TBS, were mixed 1:1 with pooled normal plasma and incubated for 30 min at 4°C, which was followed by measurement of PT, PTT-LA or dRVVT. The most pronounced effect was seen with PT (table 1). In this assay prolongation of clotting time was already seen for apple4-β_2GPI and apple4-C321S-β_2GPI at a concentration of 50 µg/ml. The observed effect was concentration dependent as shown in table 1. At a concentration of 200 µg/mL, which is the plasma concentration, apple4-β_2GPI and apple4-C321S-β_2GPI showed clotting times of 31.0 ± 4.2 and 25.8 ± 3.9 s, respectively. This corresponds for apple4-β_2GPI to a prolongation of clotting time of 15.9 s and for apple4-C321S-β_2GPI of 10.7 s. Plasma-derived β_2GPI, apple2-β_2GPI, and apple4-C321S-β_2GPI-W316S did not induce prolonged clotting times. With PTT-LA a concentration of 200 µg/mL of apple4-β_2GPI was able to prolong the clotting time from 45.2 ± 1.6 s (mean clotting time of pooled normal plasma) to 66.1 ± 8.1 s (table 2). For apple4-C321S-β_2GPI also prolongation of clotting time was observed. At a

concentration of 200 µg/mL the clotting time was 65.2 ± 13.4 s. As expected, no prolongation of clotting times was observed for plasma-derived β_2GPI, apple2-β_2GPI and apple4-C321S-β_2GPI-W316S. dRVVT showed prolonged a clotting time for apple4-β_2GPI and apple4-C321S-β_2GPI with 57.1 ± 5.9 and 56.2 ± 6.3 s, respectively at a concentration of 200 µg/mL (table 2). The mean clotting time of pooled normal plasma using dRVVT was 44.5 ± 1.7 s. Plasma-derived β_2GPI, apple2-β_2GPI, and apple4-C321S-β_2GPI were not able to induce prolongation of clotting times. So, these results suggest that dimeric apple-β_2GPI chimers were able to induce LAC activity.

To confirm that the increased clotting time were caused by the binding of dimeric apple-β_2GPI chimers to the catalytic phospholipids, correction experiments were done. Here, extra phospholipids were added to increasing concentrations of apple4-β_2GPI or apple4-C321S-β_2GPI (50-200 µg/mL) diluted in TBS, which was afterwards mixed 1:1 with pooled normal plasma and incubated for 30 min at 4ºC. After this incubation PT was measured. As shown in table 1, the prolonged clotting times disappeared by the addition of extra phospholipids. For both apple4-β_2GPI and apple4-C321S-β_2GPI clotting time was reduced to normal values at protein concentrations of 50 and 100 µg/mL. At a concentration of 200 µg/mL, clotting times were reduced to 25.1 ± 6.4 for apple4-β_2GPI and 17.8 ± 0.9 s for apple4-C321S-β_2GPI. This was a reduction of 60 % and 72% for apple4-β_2GPI and apple4-C321S-β_2GPI, respectively. This strongly suggests that the prolonged clotting times were caused by the binding of dimeric apple-β_2GPI fusion proteins to phospholipids.

TABLE 1. **Effect of apple-β_2GPI fusion proteins on clotting assays.** Plasma-derived β_2GPI and apple-β_2GPI chimers were diluted 1:1 with pooled normal plasma (mean PT 15.1 ± 1.2 s, n=20), with or without the addition of extra phospholipids, followed by the measurement of the PT. Results represent mean clotting time ± SD in s (n=3). Samples with a clotting time ≥ 18.1 s (mean PT + 20%) were considered to be positive.

chimer	PT			PT + phospholipids		
	50*	100	200	50	100	200
β_2GPI	17.4 ± 0.7	16.3 ± 0.7	16.7 ± 0.9	n.d.	n.d.	n.d.
apple2-β_2GPI	15.6 ± 1.9	15.7 ± 1.0	15.8 ± 1.5	n.d.	n.d.	n.d.
apple4-β_2GPI	18.8 ± 0.1#	25.0 ± 3.9#	31.0 ± 4.2#	15.3 ± 1.2	17.5 ± 1.8	25.1 ± 6.4#
apple4-C321S-β_2GPI	18.3 ± 1.0#	21.1 ± 1.3#	25.8 ± 0.8#	14.0 ± 0.4	14.6 ± 0.2	17.8 ± 0.9
apple4-C321S-β_2GPI-W316S	15.9 ± 0.9	15.0 ± 0.9	14.8 ± 0.5	n.d.	n.d.	n.d.

* protein concentration (µg/mL)
\# considered to be positive
n.d. not done

TABLE 2. **Effect of apple-β_2GPI fusion proteins on clotting assays.** Plasma-derived β_2GPI and apple-β_2GPI chimers were diluted 1:1 with pooled normal plasma (mean dRVVT 44.5 ± 1.7 s and mean PTT-LA 45.2 ± 1.6 s, n=15) followed by the measurement of dRVVT or PTT-LA. Data represent mean ± SD (n=3). Samples with clotting times ≥ 53.4 s for dRVVT and ≥ 54.2 s for PTT-LA were considered to be positive (mean clotting time pooled normal plasma + 20%).

chimer	dRVVT			PTT-LA		
	50*	100	200	50	100	200
β_2GPI	45.4 ± 1.4	45.9 ± 0.6	45.8 ± 1.9	43.6 ± 2.0	45.8 ± 1.0	48.0 ± 3.2
apple2-β_2GPI	44.8 ± 1.9	46.2 ± 2.9	45.4 ± 2.4	46.1 ± 1.4	44.3 ± 0.5	42.7 ± 6.2
apple4-β2GPI	50.2 ± 1.7	52.4 ± 3.6	57.1 ± 5.9#	49.8 ± 2.6	57.4 ± 3.6#	66.1 ± 8.1#
apple4-C321S–β_2GPI	49.7 ± 4.7	51.4 ± 2.8	56.2 ± 6.3#	50.5 ± 3.9	50.3 ± 2.8	65.2 ±13.4#
apple4-C321S–β_2GPI-W316S	44.5 ± 2.0	42.9 ± 1.9	43.8 ± 1.9	43.5 ± 1.7	43.7 ± 1.8	46.7 ± 4.9

* protein concentration (µg/mL)
\# considered to be positive

To determine if addition of LAC-inducing monoclonal antibodies against β_2GPI were able to induce extra prolongation of clotting time in the presence of apple4-β_2GPI or apple4-C321S-β_2GPI, coagulation assays using PT were performed. Two different monoclonal anti-β_2GPI antibodies were used: 19H9, which is directed against domain four and 23H9, which is directed against domain two of β_2GPI. First, the effect of the monoclonal anti-β_2GPI antibodies, at a concentration of 100 µg/mL, on clotting time in β_2GPI-deficient plasma was determined. Both antibodies did not prolong clotting time, as was expected (fig. 4). Second, the effect of the monoclonal antibodies (concentration 100 µg/mL), in the presence of plasma-derived β_2GPI or apple-β_2GPI fusion proteins (concentration of 200 µg/mL), on clotting time in β_2GPI-deficient plasma was determined. As shown in fig. 4, both monoclonal anti-β_2GPI antibodies were able to prolong clotting time in the presence of plasma-derived β_2GPI or apple2-β_2GPI, indicating that both monoclonal antibodies indeed were able to induce LAC activity. In the presence of apple4-β_2GPI or apple4-C321S-β_2GPI, the monoclonal anti-β_2GPI antibodies were able to induce an extra prolongation of clotting time. For apple4-β_2GPI clotting time was 48.7 s in the absence of a monoclonal antibody and 74.9 and 93.3 s in the presence of 19H9 and 23H9, respectively. Addition of apple4-C321S-β_2GPI showed a clotting time of 40.0 s, which was prolonged to 72.1 and 62.2 s when 19H9 and 23H9 were added, respectively. When the Fab fragment of monoclonal antibody 23H9 was added (concentration 100 µg/mL) no prolongation of clotting time was observed compared to the clotting time in the presence of 200 µg/mL plasma-derived β_2GPI or apple-β_2GPI fusion proteins (fig. 4). These observations suggest that multimerization of apple-β_2GPI chimeric dimers induces an extra prolongation of clotting time.

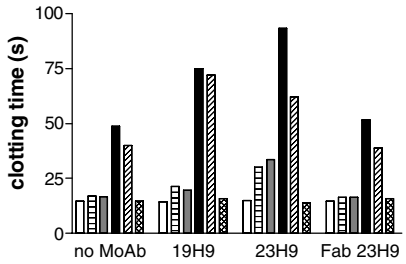

FIG. 4. **Effect of addition of monoclonal anti-β_2GPI antibodies on clotting time.** No protein (white bar), plasma-derived β_2GPI (lined bar), apple2-β_2GPI (grey bar), apple4-β_2GPI (black bar), apple4-C321S-β_2GPI (shaded bar), or apple4-C321S-β_2GPI (hatched bar) were added to β_2GPI-deficient plasma in the absence (no MoAb) or in the presence (19H9 and 23H9) of anti-β_2GPI monoclonal antibodies or the Fab fragment (Fab 23H9) of the monoclonal anti-β_2GPI antibody 23H9. This was followed by measurement of PT.

Discussion

In the antiphospholipid syndrome persistently elevated levels of antiphospholipid antibodies can be detected. These antibodies are in most cases directed against phospholipid-binding proteins such as β_2GPI and prothrombin [34-37]. It is known that when β_2GPI interacts with anti-β_2GPI-antibodies it acquires a much higher affinity for phospholipid membranes. One of the postulated mechanisms of action for anti-β_2GPI-antibodies is that when anti-β_2GPI-antibodies interact with β_2GPI, β_2GPI is able to form bivalent complexes with these antibodies, which results in a strongly increased affinity for phospholipid membranes [51-53]. Another explanation is that β_2GPI undergoes a conformational change when it interacts with such antibodies, resulting also in a strongly increased affinity for phospholipid membranes [45-49]. Here, the role of bivalent β_2GPI complexes, without the intervention of antibodies, is determined by the construction of dimeric chimers of the dimerization domain (apple4) of coagulation factor XI and β_2GPI. Introduction of the apple4 domain at the N-terminus of β_2GPI resulted in the formation of a covalent dimer of β_2GPI. A non-covalent dimer was made by fusion of a mutated apple4 domain, apple4-C321S, to β_2GPI. Gel filtration studies demonstrated that both recombinant chimers formed dimers, as had also been shown for tPA [56]. As controls, apple2-β_2GPI, a monomer, and apple4-C321S-β_2GPI-W316S, in which β_2GPI-W316S is not able to bind to phospholipids, were made. We showed that dimeric apple-β_2GPI fusion proteins bind with a 35 times increased affinity to phospholipid vesicles (fig. 2). Apple4-β_2GPI dimers bind also spontaneously to collagen type III adhered platelets and dimeric apple-β_2GPI chimers were able to induce LAC activity when added to normal plasma. Thus, the *in vitro* observed effects of anti-β_2GPI antibodies can be explained by dimerization of β_2GPI.

For apple4-β_2GPI, half maximal binding to 20% PS/80% PC vesicles was achieved at a 35-fold lower concentration than for plasma-derived β_2GPI, which indicates a strong increase of affinity for phospholipids of the dimeric apple-β_2GPI chimers. Half maximal binding for the dimeric chimers apple4-β_2GPI and apple4-C321S-β_2GPI to 20% PS/80% PC vesicles was achieved at concentrations of 0.9 and 3.7 µg/mL, respectively (fig. 2A). The difference in concentrations for half maximal binding between these two chimeric proteins could be caused

by the fact that apple4-C321S-β_2GPI is a non-covalently associated dimer, which dissociates more easily than the covalently associated dimer apple4-β_2GPI. No large differences between binding of plasma-derived β_2GPI and apple-β_2GPI fusion proteins to phospholipid vesicles with and without PE were observed, since half maximal binding of apple4-β_2GPI was achieved at 0.9 and 0.3 μg/mL for 20% PS/80% PC and 20% PS/40% PC/40% PE vesicles, respectively. The binding of β_2GPI to negatively charged phospholipids appears to be relatively independent of the presence of PE. This finding is in contrast with the observation that PE plays an important role in the inhibition of activated protein C activity by antiphospholipid antibodies [62]. β_2GPI does not discriminate between PE-containing and PE-free phospholipid surfaces and therefore inhibits pro- and anticoagulant reactions independent of the composition of the negatively charged phospholipid surfaces.

The dimeric chimers were able to induce LAC activity without the intervention of anti-β_2GPI antibodies. Three different coagulation assays were used, an activate partial thromboplastin time-based assay, a prothrombin time-based assay and a dilute Russell's viper venom time. The most responsive coagulation assay was the prothrombin time, which showed a doubling of the clotting time. The difference in responsiveness of the different coagulation assays may be the result of different sensitivities of the assays for our dimeric chimers, for instance due to differences in phospholipid composition of the tests. Since in these clotting assays a plasma system and in the phospholipid binding assays a purified system is used, the effects of the dimeric apple-β_2GPI constructs are lower in the coagulation assays. When the results of the coagulation assays are compared to what is observed in patients, the results are quite impressive. A patient with a clotting time that is 20% prolonged is considered to be LAC positive when no other abnormalities are observed (e.g. coagulation factor deficiencies). A patient with a clotting time that is doubled is considered to be strongly LAC positive. Extra prolongation of clotting time was achieved by the addition of monoclonal anti-β_2GPI antibodies, which are able to induce LAC activity by themselves, in the presence of apple-β_2GPI chimeric dimers. When the Fab fragment of monoclonal anti-β_2GPI-antibody 23H9 was added no prolongation of clotting time was observed. To all probability, the effect of the antibodies is caused by multimerization of the dimeric apple-β_2GPI fusion proteins. The difference between dimerization and multimerization of β_2GPI may be an explanation for the observed differences in LAC activities found in patients. When weak LAC activity is observed, a patient may have monoclonal anti-β_2GPI-antibodies that dimerize β_2GPI. When strong LAC activity is observed, a patient may have polyclonal anti-β_2GPI-antibodies directed towards different epitopes, which makes multimerization of β_2GPI possible.

With the recombinant dimeric β_2GPI constructs, it will be possible to discriminate between cellular activation caused by the interaction of an antibody with a FcγRII receptor and by dimerization of β_2GPI caused by binding of the antibody to β_2GPI. In this way, the dimeric apple-β_2GPI fusion proteins will be very helpful to use in animal models to investigate whether dimerization of β_2GPI explains also the *in vivo* observed complications.

In conclusion, we showed that dimerization itself is enough to mimic the effect of anti-β_2GPI antibody-β_2GPI complexes *in vitro*. This indicates that a conformational change of β_2GPI is not needed to induce an increased affinity of β_2GPI for phospholipids. We cannot exclude, however, that a conformational change of β_2GPI can also induce an increased affinity of β_2GPI for phospholipids.

References

1. Kristensen T, Schousboe I, Boel E, Mulvihill EM, Hansen RR, Moller KB, Moller NPH, Sottrup-Jensen L. Molecular cloning and mammalian expression of human beta2-glycoprotein I cDNA. *FEBS Lett* 1991; 289: 183-186.
2. Steinkasserer A, Barlow PN, Willis AC, Kertesz Z, Campbell ID, Sim RB, Norman DG. Activity, disulphide mapping and structural modelling of the fifth domain of human beta 2-glycoprotein I. *FEBS Lett* 1992; 313: 193-197.
3. Lozier J, Takahashi N, Putnam FW. Complete amino acid sequence of human plasma beta 2-glycoprotein. *Proc Natl Acad Sci USA* 1984; 81: 3640-3644.
4. Kato H, Enjyoji K. Amino acid sequence and location of the disulfide bonds in bovine β2 glycoprotein I: The presence of five sushi domains. *Biochemistry* 1991; 30: 11687-11694.
5. Bendixen E, Halkier T, Magnusson S, Sottrup-Jensen L, Kristensen T. Complete primary structure of bovine β2-glycoprotein I: Localization of the disulfide bridges. *Biochemistry* 1992; 31: 3611-3617.
6. Hunt JE, Simpson RJ, Krilis SA. Identification of a region of β_2-glycoprotein I critical for lipid binding and anti-cardiolipin antibody cofactor activity. *Proc Natl Acad Sci USA* 1993; 90: 2141-2145.
7. Hunt J, Krilis S. The fifth domain of β_2-glycoprotein I contains a phospholipid binding site (Cys281-Cys288) and a region recognized by anticardiolipin antibodies. *J Immunol* 1994; 152: 653-659.
8. Sheng YH, Sali A, Herzog H, Lahnstein J, Krilis SA. Site-directed mutagenesis of recombinant human β_2-glycoprotein I identifies a cluster of lysine residues that are critical for phospholipid binding and anti-cardiolipin antibody activity. *J Immunol* 1996; 157: 3744-3751.
9. Horbach DA, Van Oort E, Tempelman MJ, Derksen RHWM, De Groot PhG. The prevalence of a non-phospholipid-binding form of β_2-glycoprotein I in human plasma. Consequences for the development of anti-β_2-glycoprotein I antibodies. *Thromb Haemost* 1998; 80: 791-797.
10. Mehdi H, Naqvi A, Kamboh MI. A hydrophobic sequence at position 313-316 (Leu-Ala-Phe-Trp) in the fifth domain of apolipoprotein H (β_2-glycoprotein I) is crucial for cardiolipin binding. *Eur J Biochem* 2000; 267: 1770-1776.
11. Bouma B, De Groot PhG, van den Elsen JMH, Ravelli RBG, Schouten A, Simmelink MJA, Derksen RHWM, Kroon J, Gros P. Adhesion mechanism of human β2-glycoprotein I to phospholipids based on its crystal structure. *EMBO J* 1999; 18: 5166-5174.
12. Schousboe I. β_2-glycoprotein I: A plasma inhibitor of the contact activation of the intrinsic blood coagulation pathway. *Blood* 1985; 66: 1086-1091.
13. Nimpf J, Bevers EM, Bomans PHH, Till U, Wurm H, Kostner GM, Zwaal RFA. Prothrombinase activity of human platelets is inhibited by beta2-glycoprotein-I. *Biochim Biophys Acta* 1986; 884: 142-149.
14. Shi W, Chong BH, Hogg PJ, Chesterman CN. Anticardiolipin antibodies block the inhibition by β_2-glycoprotein I of the factor Xa generating activity of platelets. *Thromb Haemost* 1993; 70: 342-345.

15. Keeling DM, Wilson AJG, Mackie IJ, Isenberg DA, Machin SJ. Role of β_2-glycoprotein I and anti-phospholipid antibodies in activation of protein C in vitro. *J Clin Pathol* 1993; 46: 908-911.
16. Matsuda J, Gohchi K, Kawasugi K, Gotoh M, Saitoh N, Tsukamoto M. Inhibitory activity of anti-β_2-glycoprotein I antibody on factor Va degradation by activated-protein C and its cofactor protein S. *Am J Hematol* 1995; 49: 89-91.
17. Schousboe I, Rasmussen MS. Synchronized inhibition of the phospholipid mediated autoactivation of factor XII in plasma by β_2-glycoprotein I and anti- β_2-glycoprotein I. *Thromb Haemost* 1995; 73: 798-804.
18. Mori T, Takeya H, Nishioka J, Gabazza EC, Suzuki K. β_2-Glycoprotein I modulates the anticoagulant activity of activated protein C on the phospholipid surface. *Thromb Haemost* 1996; 75: 49-55.
19. Ieko M, Yasukouchi T, Sawada K-I, Koike T. The influence of β2-glycoprotein I on tissue factor activity. *Semin Thromb Hemost* 1998; 24: 211-215.
20. Dahlback B, Nilsson IM, Frohm B. Inhibition of platelet prothrombinase activity by a lupus anticoagulant. *Blood* 1983; 62: 218-225.
21. Nimpf J, Wurm H, Kostner GM. Interaction of β_2-glycoprotein-I with human blood platelets: influence upon the ADP-induced aggregation. *Thromb Haemost* 1985; 54: 397-401.
22. Schousboe I. Binding of beta 2-glycoprotein I to platelets: effect of adenylate cyclase activity. *Thromb Res* 1980; 19: 225-237.
23. Forastiero RR, Martinuzzo M, Carreras LO, Maclouf J. Anti-β_2-glycoprotein I antibodies and platelet activation in patients with antiphospholipid antibodies: association with increased excretion of platelet-derived thromboxane urinary abnormalities. *Thromb Haemost* 1998; 79: 42-45.
24. Chonn A, Semple SC, Cullis PR. β 2-Glycoprotein I is a major protein associated with very rapidly cleared liposomes *in vivo*, suggesting a significant role in the immune clearance of "non-self" particles. *J Biol Chem* 1995; 270: 25845-25849.
25. Wurm H. Beta 2-glycoprotein-I (apolipoprotein H) interactions with phospholipid vesicles. *Int J Biochem* 1984; 16: 511-515.
26. Hasunuma Y, Matsuura E, Makita Z, Katahira T, Nishi S, Koike T. Involvement of β_2-glycoprotein I and anticardiolipin antibodies in oxidatively modified low-density lipoprotein uptake by macrophages. *Clin Exp Immunol* 1997; 107: 569-573.
27. Balasubramanian K, Chandra J, Schroit A. Immune clearance of phosphatidylserine-expressing cells by phagocytes. The role of β2-glycoprotein I in macrophage recognition. *J Biol Chem* 1997; 272: 31113-31117.
28. Wilson WA, Gharavi AE, Koike T, Lockshin MD, Branch DW, Piette JC, Brey R, Derksen RHWM, Harris EN, Hughes GRV, Triplett DA, Khamashta MA. International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: report of an international workshop. *Arthritis Rheum* 1999; 42: 1309-1311.
29. Love PE, Santoro SA. Antiphospholipid antibodies: anticardiolipin and the lupus anticoagulant in systemic lupus erythematosus (SLE) and in non-SLE disorders. Prevalence and clinical significance. *Ann Intern Med* 1990; 112: 682-698.
30. Roubey RAS. Autoantibodies to phospholipid-binding plasma proteins: a new view

of lupus anticoagulants and other "antiphospholipid" autoantibodies. *Blood* 1994; 84: 2854-2867.
31. Harris EN, Asherson RA, Hughes GR. Antiphospholipid antibodies-autoantibodies with a difference. *Annu Rev Med* 1988; 39: 261-271.
32. De Groot PhG, Oosting JD, Derksen RHWM. Antiphospholipid antibodies: specificity and pathophysiology. *Bailliere's Clin Haematol* 1993; 6: 691-709.
33. Horbach DA, Van Oort E, Donders RCJM, Derksen RHWM, De Groot PG. Lupus anticoagulant is the strongest risk factor for both venous and arterial thrombosis in patients with systemic lupus erythematosus - Comparison between different assays for the detection of antiphospholipid antibodies. *Thromb Haemost* 1996; 76: 916-924.
34. Galli M, Comfurius P, Maassen C, Hemker HC, de Baets MH, van Breda-Vriesman PJC, Barbui T, Zwaal RFA, Bevers EM. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein factor. *Lancet* 1990; 335: 1544-1547.
35. McNeil HP, Simpson RJ, Chesterman CN, Krilis SA. Antiphospholipid antibodies are directed against a complex antigen that includes a lipid binding inhibitor of coagulation: β_2-glycoprotein I (apolipoprotein H). *Proc Natl Acad Sci USA* 1990; 87: 4120-4124.
36. Brandt JT. Antibodies to b_2-glycoprotein I inhibit phospholipid dependent coagulation reactions. *Thromb Haemost* 1993; 70: 598-602.
37. Bevers EM, Galli M, Barbui T, Comfurius P, Zwaal RFA. Lupus anticoagulant IgG's (LA) are not directed to phospholipids only, but to a complex of lipid-bound human prothrombin. *Thromb Haemost* 1991; 66: 629-632.
38. Loizou S, McCrea JD, Rudge A, Reynolds.R., Boyle CC, Harris EN. Measurement of anti-cardiolipin antibodies by an enzyme-linked immunosorbent assay (ELISA): standardization and quantitation of results. *Clin Exp Immunol* 1985; 62: 738-745.
39. Harris EN, Gharavi AE, Boey ML, Patel BM, Mackworth-Young CG, Loizou S, Hughes GRV. Anticardiolipin antibodies: detection by radio-immunoassay and association with thrombosis in systemic lupus erythematosus. *Lancet* 1983; 2: 1211-1214.
40. Exner T, Triplett DA, Taberner D, Machin SJ. Guidlines for testing and revised criteria for lupus anticoagulants. *Thromb Haemost* 1991; 65: 320-322.
41. Brandt JT, Triplett DA, Alving B, Scharrer I. Criteria for the diagnosis of lupus anticoagulants: An update. *Thromb Haemost* 1995; 74: 1185-1190.
42. Permpikul P, Rao LVM, Rapaport SI. Functional and binding studies of the roles of prothrombin and β2-glycoprotein I in the expression of lupus anticoagulant activity. *Blood* 1994; 83: 2878-2892.
43. Oosting JD, Derksen RHWM, Entjes HTI, Bouma BN, De Groot PhG. Lupus anticoagulant activity is frequently dependent on the presence of β_2-glycoprotein I. *Thromb Haemost* 1992; 67: 499-502.
44. Roubey RAS, Pratt CW, Buyon JP, Winfield JB. Lupus anticoagulant activity of autoimmune antiphospholipid antibodies is dependent upon beta 2-glycoprotein I. *J Clin Invest* 1992; 90: 1100-1104.
45. Wagenknecht DR, McIntyre JA. Changes in β_2-glycoprotein I antigenicity induced by phospholipid binding. *Thromb Haemost* 1993; 69: 361-365.
46. Ichikawa K, Khamashta MA, Koike T, Matsuura E, Hughes GRV. β_2-Glycoprotein I reactivity of monoclonal anticardiolipin antibodies from patients with the

antiphospholipid syndrome. *Arthritis Rheum* 1994; 37: 1453-1461.
47. Pengo V, Biasiolo A, Fior MG. Autoimmune antiphospholipid antibodies are directed against a cryptic epitope expressed when β2-glycoprotein I is bound to a suitable surface. *Thromb Haemost* 1995; 73: 29-34.
48. Chamley LW, Duncalf AM, Konarkowska B, Mitchell MD, Johnson PM. Conformationally altered β2-glycoprotein I is the antigen for anti-cardiolipin autoantibodies. *Clin Exp Immunol* 1999; 115: 571-576.
49. Galazka M, Tang M, DeBari VA, Kohles JD, Lee JK, Keil LB, Petersheim M. Modification of β2-glycoprotein I by glutardialdehyde. Conformational changes and aggregation accompany exposure of cryptic autoepitope. *Appl Biochem Biotech* 1999; 76: 1-13.
50. Wang S-X, Sun Y-T, Sui S-F. Membrane-induced conformational change in human apolipoprotein H. *Biochem J* 2000; 348: 103-106.
51. Willems GM, Janssen MP, Pelsers MAL, Comfurius P, Galli M, Zwaal RFA, Bevers EM. Role of divalency in the high-affinity binding of anticardiolipin antibody-β_2-glycoprotein I complexes to lipid membranes. *Biochemistry* 1996; 35: 13833-13842.
52. Arnout J, Wittevrongel C, Vanrusselt M, Hoylaerts M, Vermylen J. Beta-2-glycoprotein I dependent lupus anticoagulants form stable bivalent antibody beta-2-glycoprotein I complexes on phospholipid surfaces. *Thromb Haemost* 1998; 79: 79-86.
53. Sheng Y, Kandiah DA, Krilis SA. Anti-β_2-glycoprotein I autoantibodies from patients with the "antiphospholipid" syndrome bind to β_2-glycoprotein I with low affinity: dimerization of β_2-glycoprotein I induces a significant increase in anti-b_2-glycoprotein I antibody affinity. *J Immunol* 1998; 161: 2038-2043.
54. Takeya H, Mori T, Gabazza EC, Kuroda K, Deguchi H, Matsuura E, Ichikawa K, Koike T, Suzuki K. Anti-β2-glycoprotein I (β2GPI) monoclonal antibodies with lupus anticoagulant-like activity enhance the β2GPI binding to phospholipids. *J Clin Invest* 1997; 99: 2260-2268.
55. Meijers JCM, Davie EW, Chung DW. Expression of human blood coagulation factor XI: Characterization of the defect in factor XI type III deficiency. *Blood* 1992; 79: 1435-1440.
56. Meijers JC, Mulvihill ER, Davie EW, Chung DW. Apple four in human blood coagulation factor XI mediates dimer formation. *Biochemistry* 1992; 31: 4684.
57. Brunner J, Skrabal P, Hauser H. Single bilayer vesicles prepared without sonication. Physio-chemical properties. *Biochim Biophys Acta* 1976; 455: 322-331.
58. Van Wijnen M, Stam JG, Van't Veer C, Meijers JCM, Reitsma PH, Bertina RM, Bouma BN. The interaction of protein S with the phospholipid surface is essential for the activated protein C-independent activity of protein S. *Thromb Haemost* 1996; 76: 397-403.
59. Rouser G, Fkeischer S, Yamamoto A. Two dimensional thin layer chromatography separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* 1970; 5: 494-496.
60. van Zanten HG, Saelman EUM, Schut Hese KM, Wu YP, Slootweg PJ, Nieuwenhuis HK, De Groot PG, Sixma JJ. Platelet adhesion to collagen type IV under flow conditions. *Blood* 1996; 88: 3862-3871.
61. Heemskerk JWM, Vuist WMJ, Feijge MAH, Reutelingsperger CPM, Lindhout T.

Collagen but not fibrinogen surfaces induce bleb formation, exposure of phosphatidylserine, and procoagulant activity of adherent platelets: evidence for regulation by protein tyrosine kinase-dependent Ca^{2+} responses. *Blood* 1997; 90: 2615-2625.

62. Smirnov MD, Triplett DA, Comp PC, Esmon NL, Esmon CT. On the role of phosphatidylethanolamine in the inhibition of activated protein C activity by antiphospholipid antibodies. *J Clin Invest* 1995; 95: 309-316.

Chapter 4

An explanation for arterial thrombosis in the antiphospholipid syndrome: Dimers of β_2-glycoprotein I increase platelet deposition to collagen via interaction with phospholipids and a cellular receptor

Bianca C.H. Lutters[1,3], Ronald H.W.M. Derksen[2], Winnie L.Tekelenburg[1], Peter J. Lenting[1,3], Jef Arnout[4], Philip G. de Groot[1,3]

Departments of [1]Haematology and [2]Rheumatology and Clinical Immunology, University Medical Center, Utrecht and [3]Institute of Biomembranes, Utrecht University, [4]Center for Molecular and Vascular Biology, University of Leuven, Leuven, Belgium.

Abstract

Patients with prolonged clotting times caused by lupus anticoagulant (LAC) are at risk for thrombosis. This paradoxal association is not understood. LAC is frequently caused by anti-β_2-glycoprotein I (β_2GPI) antibodies. Antibody-induced dimerization of β_2GPI increases the affinity of β_2GPI for phospholipids, explaining the observed prolonged clotting times. We constructed dimers of β_2GPI which mimic effects of β_2GPI-anti-β_2GPI antibody complexes and studied their effects on platelet adhesion and thrombus formation in a flow system.

Dimeric β_2GPI increased platelet adhesion to collagen with 150% and increased the number of large aggregates. We also observed increased platelet adhesion to collagen when whole blood was spiked with patient-derived polyclonal anti-β_2GPI or some, but not all, monoclonal anti-β_2GPI antibodies with LAC activity. These effects could be abrogated by inhibition of thromboxane synthesis. A LAC positive monoclonal anti-β_2GPI antibody, which did not affect platelet adhesion, prevented the induced increase in platelet adhesion by β_2GPI dimers. Furthermore, increased platelet adhesion disappeared after preincubation with receptor-associated protein (RAP), a universal inhibitor of interaction of ligands with members of the low-density lipoprotein (LDL) receptor family. We show that dimeric β_2GPI induces increased platelet adhesion and thrombus formation, which depends on the activation of a member of the LDL-receptor family.

Introduction

β_2-glycoprotein I (β_2GPI), also known as apolipoprotein H, is a single chain protein, which consists of 326 amino acids and has a molecular weight of 42 kDa. β_2GPI is present in plasma at a concentration of about 200 µg/ml. It is a member of the complement control protein (CCP) or the short consensus repeat (SCR) superfamily. β_2GPI contains 5 domains, of which the first four domains consist of ~60 amino acids and contain 2 disulfide bonds. The fifth domain is aberrant with an extra C-terminal loop and an extra disulfide bond [1-5]. This domain, in particular the regions Cys281 to Cys288 and Ser311 to Lys317, is important for phospholipid binding [6-9]. Furthermore, a polymorphism in which Trp316 is replaced by a serine, results in a completely abolished binding of β_2GPI to phospholipids [10]. The elucidation of the crystal structure of β_2GPI confirmed these observations [11,12]. β_2GPI has been suggested to influence coagulation [13-20], platelet function [21-23], lipoprotein metabolism [24-27], and apoptosis [24], however, all these observations were based on in vitro experiments. At this moment, the in vivo function of β_2GPI is not known.

Antibodies against β_2GPI [28,29] can prolong phospholipid-dependent coagulation assays, an activity called lupus anticoagulant (LAC). The presence of LAC activity is associated with thrombotic complications. This association is called the antiphospholipid syndrome [30-35]. We previously described that fusion proteins consisting of β_2GPI and the dimerization domain of coagulation factor XI, apple4, are able to mimic the in vitro effects of β_2GPI-anti-β_2GPI antibody complexes [36]. We showed that apple4-β_2GPI and apple4-C321S-β_2GPI bind with much higher affinity to phospholipid and to platelets adhered to collagen than plasma-derived β_2GPI. It was also shown that these dimers have LAC activity.

Here, we use an in vitro flow system in which whole blood is perfused over collagen or fibronectin to study effects of dimeric β_2GPI constructs and anti-β_2GPI antibodies on platelet adhesion and thrombus formation. We show that dimers of β_2GPI and anti-β_2GPI antibodies are able to sensitize platelets, which results in an increased platelet deposition on collagen containing surfaces. Our results also suggest that a member of the LDL-receptor family is involved in this phenomenon.

Materials and Methods

Proteins

Plasma-derived β_2GPI was purified from human plasma as described by Horbach et al. [34]. The chimeric dimers apple4-β_2GPI and apple4-C321S-β_2GPI were constructed as described previously [36]. As controls, the monomer apple2-β_2GPI and the dimer apple4-C321S-β_2GPI-W316, in which β_2GPI-W316S is not able to bind to phospholipids, were made. The proteins were expressed by stably transfected baby hamster kidney cells[36]. Expression of all fusion constructs was performed in conditioned serum-free medium (DMEM F12 supplemented with 1% UltroserG; Life Technologies, Inc., Paisley, U.K.). Proteins were purified using a monoclonal antibody to β_2GPI (21B2) bound to CNBr-activated Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden)[10]. Bound fusion proteins were eluted with 0.1 M glycine

β_2GPI, apple4-C321S-β_2GPI and apple4-C321S-β_2GPI-W316S containing fractions were dialyzed against 50 mM Na_2HPO_4, pH 7.0, containing 50 mM NaCl and were subjected to further purification on a mono S column using FPLC (Amersham Pharmacia Biotech, Uppsala, Sweden). Fusion proteins were eluted with a linear salt gradient from 50 mM NaCl to 1 M NaCl. All chimers were dialyzed against TBS (50 mM Tris, 150 mM NaCl, pH 7.4). Fibronectin was isolated from human plasma by affinity chromatography with gelatin-Sepharose as described previously [37]. Patient anti-β_2GPI antibodies (MB) were purified using β_2GPI coupled to CNBr-activated Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden). Bound antibodies were eluted with 3 M KCNS in TBS (50 mM Tris, 150 mM NaCl, pH 7.4) and dialyzed against TBS. Anti-Fcγ-receptor RII monoclonal antibody IV.3 (ATCC, Manassas, VA) and monoclonal anti-β_2GPI antibody 4F3 were purified from hybridoma medium using a protein G column. Bound antibodies were eluted with 0.1 M glycine, pH 2.7, neutralized with 1 M Tris, pH 9, and dialyzed against TBS. Receptor-associated protein fused to glutathione S-transferase (GST-RAP) was prepared as described previously [38]. Afterwards, the protein was filtered (0.45 µm filter). Protein concentration was determined using a bichinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) according to the instructions of the manufacturer, and with bovine serum albumin (BSA) as a standard. Monoclonal anti-β_2GPI antibodies 22A11, 23H9, 27G7, and 19H9 were described previously [36,39] and monoclonal anti-β_2GPI antibodies 1F12 and 2B2 were kindly provided by Dr. A. Tincani (Clinical Immunology Unit, Spedali Civili, Brescia, Italy).

Patients

Anti-β_2GPI antibodies were isolated from plasma from three female patients with antiphospholipid syndrome (MB, KJ and R, with informed consent). Two of these anti-β_2GPI antibodies were LAC positive after isolation (MB and R) and one antibody was LAC negative (KJ; see determination of LAC activity). Patient MB (age 32) was LAC positive and had IgG class anti-β_2GPI antibodies [34]. She had mild, chronic thrombocytopenia, cerebral infarction and recurrent first and second trimester pregnancy loss as manifestations of the antiphospholipid syndrome. Patient KJ (44 years) had lupus like disease and thrombosis of the distal aorta and patient R (age 46) had primary antiphospholipid syndrome (multiple cerebral infarctions, chronic mild thrombocytopenia and livedo reticularis). Plasma from both patient KJ and R were LAC positive, and contained anti-β_2GPI (IgG isotype) and anti-prothrombin antibodies (IgG and IgM isotype). Plasma from patient R was also positive for IgM class anti-β_2GPI antibodies.

Surfaces

Human fibronectin and fibrinogen (plasminogen, von Willebrand factor and fibronectin free; Kordia Life Sciences, Leiden, The Netherlands) were coated on Thermanox^R coverslips (Nunc, Inc., Naperville, IL, USA) at a concentration of 100 µg/mL for 1 h at room temperature. Human placental collagen type III (Sigma, St Louis, MO, USA) was solubilized overnight in 50 mmol/L acetic acid and sprayed onto Thermanox^R coverslips at a density 30 µg/mL with a retouching airbrush (model 100, Badger Brush Co). For the immunofluoresence studies glass coverslips (18 x 18 mm; Menzel Glä ser, Braunschweig, Germany) were used. After coating and spraying, the coverslips were blocked for at least 30 minutes at room

temperature with 1% human serum albumin (ICN Biomedicals, Inc., Aurora, OH, USA) in phosphate-buffered saline, pH 7.4 (PBS).

Blood collection

Freshly drawn venous blood was collected from healthy donors (with informed consent) into 1/10 of volume of 3.2% tri-sodium citrate (w/v). These donors denied to have taken aspirin or other platelet function inhibitors during the previous ten days.

Perfusate

The chimeric apple-β_2GPI proteins or plasma-derived β_2GPI were added 5 minutes before the start of the perfusion at a concentration of 100 µg/mL to whole blood and incubated at 37°C (5% of the total volume). As a control, buffer (TBS) was added to whole blood. Anti-β_2GPI antibodies were added 5 min prior to the perfusion at a concentration of 50 µg/mL to whole blood and incubated at 37°C. Indomethacin (Sigma, St Louis, MO, USA) was added at a concentration of 30 µmol/L and incubated for 30 min at room temperature prior to the addition of chimeric proteins, plasma-derived β_2GPI or anti-β_2GPI antibodies. The TxA_2 receptor antagonist and thromboxane receptor blocker SQ30741, a kind gift of Bristol-Meyers-Squibb Co (Maarssen, the Netherlands), was added at a concentration of 10 µmol/L and incubated for 30 min at room temperature before the addition of apple-β_2GPI proteins, plasma-derived β_2GPI. RAP, which inhibits the interaction of ligands with all members of the LDL-receptor family, was added at a concentration of 10 µg/mL and incubated 5 min at 37°C before addition of dimeric β_2GPI or anti-β_2GPI antibodies. Antibody IV.3, which blocks the Fcγ-receptor RII but does not activate the receptor, was added at a concentration of 1 µg/mL and incubated for 10 min at 37°C prior to the addition of anti-β_2GPI antibodies.

Perfusion studies

Perfusions were performed in a single-pass perfusion chamber under nonpulsatile flow conditions using a modified parallel plate perfusion with a slit width of 2 mm and a slit height of 0.1 mm [40]. Experiments with collagen type III as a surface were done with a perfusion time of 90 s, at a shear rate of 800 s^{-1}, which is representing the flow rate in small arteries [41]. Experiments with fibronectin as a surface were done with a perfusion time of 90 s and a shear rate of 300 s^{-1}, which is representing the flow rate in the aorta and large arteries [41]. The prewarmed blood (1.5 mL for triplicates and 1 mL for duplicates) was drawn through the perfusion chamber by an infusion pump (pump 22, model 2400-004; Harvard, Natrick, MA, USA). Afterwards, the coverslips were taken from the perfusion chamber and rinsed with HEPES-buffered saline (10 mmol/L HEPES, 150 mmol/L NaCl, pH 7.4), fixed in 0.5% glutaraldehyde in PBS, dehydrated with methanol and stained with May-Grünwald/Giemsa as previously described [37]. Platelet deposition was evaluated with a light microscope equipped with a JAI-CCD camera (Copenhagen, Denmark) coupled to a Matrox frame grabber (Matrox Electronic Systems Ltd., Quebec, Canada) using Optimas 6.2 software (Optimas Inc, Seattle, WA, USA) for image analysis. Evaluation for platelet adhesion was performed on thirty fields, perpendicular to the flow direction. Platelet adhesion was expressed as the percentage of the surface covered with platelets relative to adhesion in the presence of buffer. Thrombus size was evaluated using watershed provided by the Optimas software package. Results are

expressed as the percentage of total surface coverage present in a certain thrombus size category.

Determination of LAC activity

Coagulation assays were performed in a KC-10 coagulometer (Amelung, Lemgo, Germany). To detect LAC activity, three different coagulation assays were used: an activated partial thromboplastin time-based assay sensitized to detect LAC activity (PTT-LA; Diagnostica Stago, Asnieres-sur-Seine, France), a tissue factor-based assay using innovin (Dade Behring, Marburg, Germany), and a dilute Russell's viper venom time (dRVVT; Gradipore Ltd, North Ryde, Australia). All were measured as described previously [36]. Anti-β_2GPI antibodies were added at a concentration of 100 µg/mL to the plasma. This concentration is comparable to 50 µg/mL used in whole blood perfusions. Clotting time ratios were calculated by dividing the clotting time in the presence of antibody by the clotting time in the absence of antibody. Ratios ≥ 1.2 were considered to be positive. An anti-β_2GPI antibody was considered LAC positive when at least one of the coagulation assays was positive. As shown in table 1, all, but one (1F12), monoclonal anti-β_2GPI antibodies we used had LAC activity. Two of the monoclonal antibodies, 2B2 and 22A11, were LAC positive in three assays, and 27G7 was positive using the tissue factor-based assay and the dRVVT. The other monoclonal anti-β_2GPI antibodies, 23H9, 19H9, and 4F3, were only positive in the tissue factor-based assay. The purified patient anti-β_2GPI antibodies MB and R were LAC positive in the PTT-LA and dRVVT, respectively. Patient antibody KJ was LAC negative.

TABLE 1. **Effect of anti-β_2GPI antibodies on coagulation assays.** Anti-β_2GPI antibodies were added at a concentration of 100 µg/mL to pooled normal plasma. Afterwards a tissue factor-based assay (innovin), a PTT-LA, and a dRVVT were performed. The clotting time in the presence of antibody was divided by the clotting time in the absence of an antibody. Ratios ≥ 1.2 were considered to be positive (#).

Antibody	Innovin	PTT-LA	dRVVT	LAC
MB	1.07	1.53#	1.04	+
R	1.06	1.00	1.72#	+
KJ	1.04	1.06	1.00	-
23H9	1.88#	1.19	1.11	+
27G7	1.32#	1.16	1.56#	+
19H9	1.29#	1.19	1.10	+
22A11	1.71#	1.33#	1.26#	+
2B2	1.56#	1.43#	1.45#	+
4F3	1.50#	1.19	1.12	+
1F12	1.00	1.16	1.11	-

Immunofluoresence studies

Whole anticoagulated blood was perfused over collagen type III in the presence of different anti-β_2GPI antibodies. Binding of these antibodies to platelets was determined as follows. After perfusion, coverslips were fixed with 3% paraformaldehyde/0.005% glutaraldehyde in PBS, pH 7.4 for 30 min, washed three times with PBS, pH 7.4, and blocked for 10 min with PBS containing 1% BSA and 0.1% glycine, pH 7.4. Afterwards coverslips were incubated with a fluorescein isothiocyanate (FITC)-labeled goat-anti-mouse antibody (Becton Dickinson, San Jose, CA, USA), diluted 1:20 in PBS/1% BSA/0.1% glycine, pH 7.4 for 45 min at 37ºC., followed by three times washing with PBS. Finally, coverslips were mounted in MOWIOL 4-88 (Calbiochem, La Jolla, CA, USA) and 0.1% PPD (para-phenylene diamine, Sigma, St Louis, MO, USA).

Statistical analysis

Perfusions were performed three times in triplicate for each condition when the effects of the chimeric constructs were determined and performed three times in duplicate for each condition when the effects of the anti-β_2GPI antibodies were examined. The effect of RAP in the presence of dimeric β_2GPI was also performed three times in duplicate. The results are presented as mean ± standard deviation (SD) from experiments with blood from three different healthy donors. The difference between the various conditions was tested by analysis of variance (ANOVA) and Bonferoni was used as post-test. The results were considered significant at $p<0.05$.

Results

Effect of dimers of β_2GPI on platelet adhesion to collagen and fibronectin

To determine if apple-β_2GPI dimers have an effect on platelet adhesion we used a flow system with collagen and fibronectin as surfaces at shear rates of 800 s^{-1} and 300 s^{-1}, respectively. Whole blood was incubated with buffer or with 100 µg/ml of plasma-derived β_2GPI or apple-β_2GPI constructs prior to perfusion. Using collagen as a surface, platelet coverage after 90 s in the presence of buffer was 9.8 ± 2.0 %, which was set at 100% (fig. 1). Addition of plasma-derived β_2GPI, monomer apple2-β_2GPI, or the non-phospholipid binding dimer apple4-C321S-β_2GPI-W316S, did not affect coverage. In contrast, in the presence of the LAC-inducing dimers apple4-β_2GPI or apple4-C321S-β_2GPI, platelet adhesion levels increased to 157 ± 14% ($p<0.001$) and 148 ± 25% ($p<0.01$), respectively.

The morphology of the platelet aggregates on collagen in the presence of apple4-β_2GPI and apple4-C321S-β_2GPI was different from that with plasma-derived β_2GPI (fig. 2). Analysis of the thrombus size showed that 17% and 28% of the aggregates were larger than 300 μm^2 in the presence of apple4-β_2GPI and apple4-C321S-β_2GPI, respectively. In the presence of plasma-derived β_2GPI no increase in thrombus size was found (fig. 2D). These results indicate that the presence of the chimeric dimers apple4-β_2GPI and apple4-C321S-β_2GPI results in increased platelet adhesion and thrombus formation when whole anticoagulated blood is perfused over collagen.

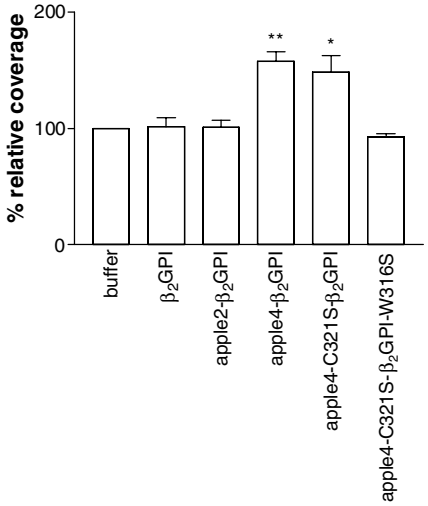

FIG. 1. **Platelet deposition on collagen type III after perfusion of whole blood in the presence of β_2GPI or dimers of β_2GPI.** Blood was perfused for 90 s at a shear rate of 800 s^{-1} and proteins were added at a concentration of 100 µg/mL. Platelet adhesion was expressed as the percentage of the surface covered with platelets relative to adhesion in the presence of buffer. Data are mean ± SD for three separate experiments. * $p<0.01$, ** $p<0.001$

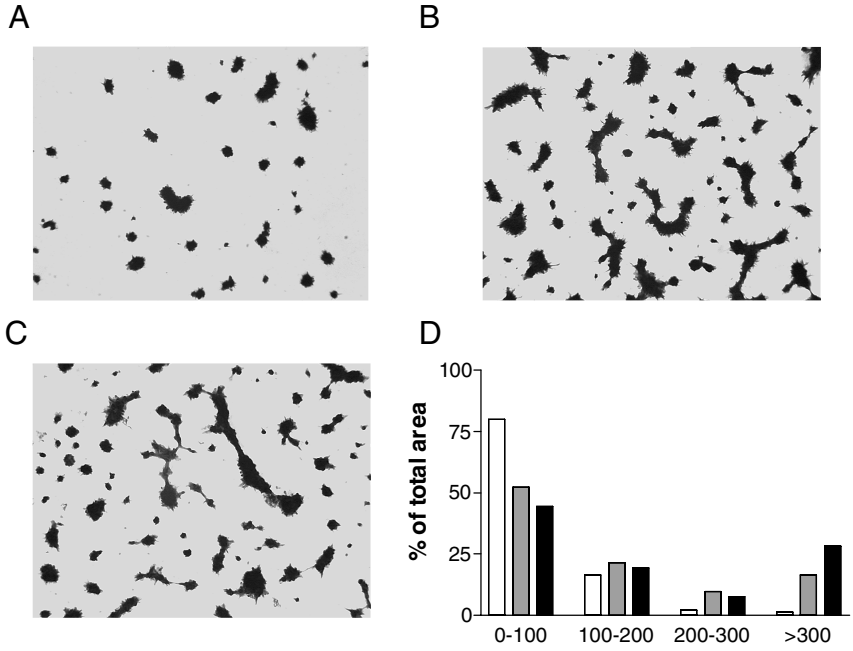

FIG. 2. **Morphology of platelet thrombi on collagen type III after perfusion of whole blood for 90 s at a shear rate of 800 s^{-1}.** Perfusions were carried out in the presence of 100 mg/mL of β_2GPI (*A*), apple4-β_2GPI (*B*) or apple4-C321S-β_2GPI (*C*). Images were made with a light microscope (40x objective). Thrombus size (in μm^2) was evaluated by watershed (*D*) showing β_2GPI (open bars), apple4-β_2GPI (gray bars), and apple4-C321S-β_2GPI (black bars).

We used fibronectin as a surface to determine if the increased platelet adhesion with apple-β_2GPI dimers was specific for collagen as a surface. Furthermore, the morphology of the adhered platelets to fibronectin is different from that adhered to collagen. Platelets are able to form aggregates on collagen, but on fibronectin they adhere more heterogeneous, showing contact platelets, spread platelets and only a few small aggregates. Using fibronectin as a surface, the adhesion level in the presence of buffer was $7.8 \pm 2.1\%$. This was set at 100%. In the presence of apple4-β_2GPI and apple4-C321S-β_2GPI, coverage increased to $222 \pm 59\%$ ($p<0.05$) and $230 \pm 57\%$ ($p<0.05$), respectively. Addition of plasma-derived β_2GPI, apple2-β_2GPI or apple4-C321S-β_2GPI-W316S had no effect on platelet adhesion. This shows that platelet adhesion to fibronectin also is increased in the presence of dimeric β_2GPI. Platelet adhesion to fibrinogen was not affected by the presence of dimeric β_2GPI (results not shown).

Role of thromboxane A_2 formation

Since patients with the antiphospholipid syndrome are described to have higher urinary levels of thromboxane B_2 (TxB_2), which is a stable metabolite of thromboxane A_2 (TxA_2), we investigated whether the effect of dimeric β_2GPI on platelet adhesion was mediated by TxA_2. Therefore, whole blood was treated with 30 µM of indomethacin for 30 min at room temperature prior to incubation with plasma-derived β_2GPI or apple-β_2GPI chimeric dimers to block TxA_2 formation in platelets. As shown in figure 3A, indomethacin had no effect on platelet adhesion in the presence of plasma-derived β_2GPI. However, preincubation with indomethacin abolished the enhancing effects of apple4-β_2GPI and apple4-C321S-β_2GPI on platelet adhesion to collagen.

Next, we preincubated whole blood with 10 µmol/L of SQ30741, a TxA_2 receptor antagonist and a thromboxane-synthase blocker, for 30 min at room temperature prior to incubation with plasma-derived β_2GPI or dimeric β_2GPI. SQ30741 had no effect on platelet adhesion in the presence of plasma-derived β_2GPI. However, it completely blocked the increased adhesion to collagen in the presence of apple4-β_2GPI and apple4-C321S-β_2GPI (fig. 3A).

Analysis of the aggregate size showed that indomethacin or SQ30741 had no effect on thrombus size in the presence of plasma-derived β_2GPI (fig. 3B). In the presence of apple4-β_2GPI, the percentage of thrombi larger than 300 μm^2 decreased from 17% in the absence of an inhibitor to 2% and 1% in the presence of indomethacin or SQ30741, respectively (fig.3C). Addition of apple4-C321S-β_2GPI to whole blood, reduced the percentage of aggregates larger than 300 μm^2 from 28% to 4% and 2% in the presence of indomethacin and SQ30741, respectively (fig.3D).

Effect of LAC positive anti-β_2GPI antibodies on platelet adhesion

To support the observations with dimer β_2GPI, we performed perfusion studies with patient-derived as well as monoclonal anti-β_2GPI antibodies. Platelet coverage noted with whole anticoagulated blood that was perfused over collagen in the presence of buffer was set at 100%. Addition of 50 µg/mL of the LAC positive patient-derived anti-β_2GPI antibodies MB and R to whole blood, which is comparable to 100 µg/mL in plasma as used in the LAC assays, increased platelet adhesion to $135 \pm 9\%$ and $133 \pm 10\%$, respectively (table 2). In the presence of monoclonal antibodies (50 µg/mL), 23H9, 27G7, 19H9, and 22A11 coverage increased

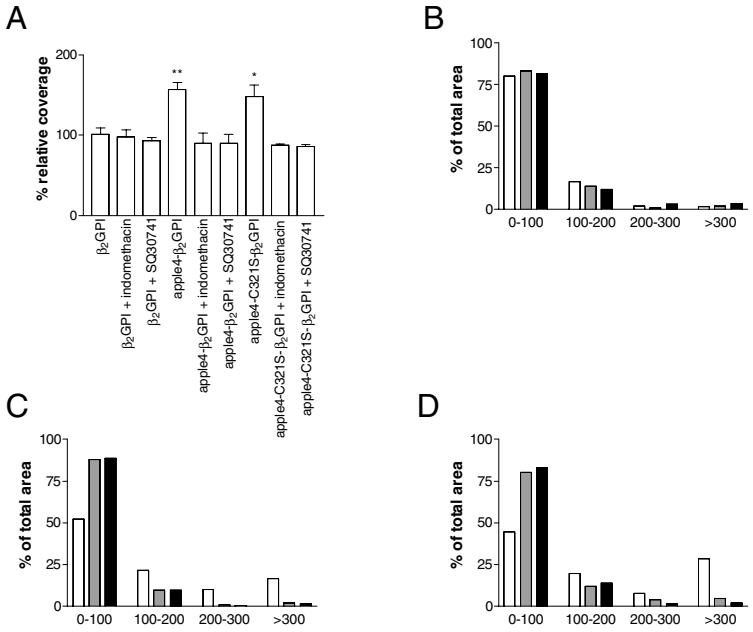

FIG. 3. **Perfusion over collagen type III in the presence of indomethacin or SQ30741.** Whole blood was incubated with indomethacin (30 μM) or SQ30741 (10 mM) for 30 min at room temperature, followed by an incubation of 5 min with 100 μg/mL of β_2GPI, apple4-β_2GPI, or apple4-C321S-β_2GPI at 37℃. Afterwards, blood was perfused at a shear rate of 800 s^{-1} for 90 s. Platelet deposition was expressed as the percentage of the surface covered with platelets relative to adhesion in the presence of buffer. Data are mean ± SD for three separate experiments. (*A*). Thrombus size (in μm^2) in the presence of β_2GPI (*B*), apple4-β_2GPI (*C*), or apple4-C321S-β_2GPI (*D*) was evaluated by watershed. Aggregate size in the absence of an inhibitor (open bars), with indomethacin (gray bars) and with SQ30741 (black bars) is indicated. * $p<0.01$, ** $p<0.001$

significantly. In the presence of LAC positive monoclonal antibodies 2B2, 4F3, and the LAC negative patient-derived antibody KJ and the LAC negative monoclonal antibody 1F12 no increase in platelet adhesion was observed (table 2). These results show that the presence of most, but not all LAC positive anti-β_2GPI antibodies results in increased platelet coverage on collagen. A LAC negative anti-β_2GPI antibody did not affect platelet adhesion. This suggests that presence of LAC activity by itself is not the only parameter that is important in the increased platelet adhesion to collagen in this flow system. Therefore, we measured binding of the anti-β_2GPI antibodies to platelets in the perfusion system. Coverslips were fixed after perfusion and binding was determined using immunofluoresence. As shown in table 2, the LAC negative anti-β_2GPI antibody did not to bind to platelets, whereas all monoclonal LAC positive anti-β_2GPI antibodies did bind to the platelets.

To test if the increase in platelet coverage found in the presence of anti-β_2GPI antibodies also was mediated by TxA_2, we performed perfusion studies in the presence of indomethacin. As shown in figure 4, pre-incubation with indomethacin completely abolished

the increased platelet adhesion to collagen in the presence of the monoclonal anti-β_2GPI antibody 22A11 or the purified patient anti-β_2GPI antibody MB. This shows that anti-β_2GPI antibodies, just like dimers of β_2GPI, are able to induce an increase in platelet adhesion to collagen, which needs the formation of TxA_2.

TABLE 2. **Effects of anti-β_2GPI antibodies in an in vitro perfusion system.** Whole anticoagulated blood was incubated for 5 min at 37°C with anti-β_2GPI antibodies. Blood was perfused at a shear rate of 800 s^{-1} for 90 s. Platelet adhesion was expressed as the percentage of the surface covered with platelets relative to adhesion in the presence of buffer. Data are mean ± SD for three separate experiments. Binding of the antibodies to the platelets was detected with immunofluoresence.

Antibody	Relative platelet coverage (%)	Binding to platelets #
buffer	100	
MB	135 ± 9 **	n.d !
R	133 ± 10 **	n.d.
KJ	101 ± 5	n.d.
23H9	157 ± 13 **	+
27G7	156 ± 28 **	+
19H9	180 ± 7 ***	+
22A11	132 ± 14 *	+
2B2	99 ± 3	+
4F3	107 ± 4	+
1F12	113 ± 8	-

* p<0.05
** p<0.01
*** p<0.001
\# + indicates binding to platelets present, - binding to platelets absent
! not determined

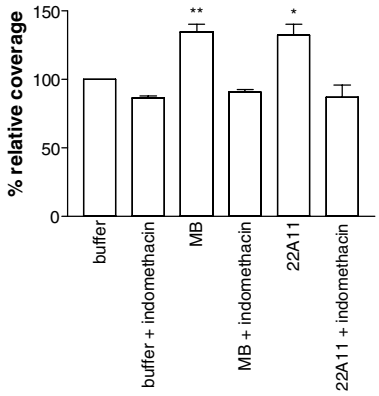

FIG. 4. **Perfusion over collagen type III in the presence of anti-β_2GPI antibodies (50 µg/mL).** Whole anticoagulated blood was incubated for 5 min at 37°C with anti-β_2GPI antibodies in the absence or the presence of indomethacin. Blood was perfused at a shear rate of 800 s^{-1} for 90 s. Platelet adhesion was expressed as the percentage of the surface covered with platelets relative to adhesion in the presence of buffer. Data are mean ± SD for three separate experiments. * $p < 0.05$, ** $p<0.01$

Role of a receptor

An explanation for our results is that after binding, dimeric β_2GPI interacts with other structures, probably a receptor, on the platelet membrane and that this interaction is blocked by monoclonal anti-β_2GPI antibodies 2B2 and 4F3. Therefore, we performed an experiment in which we tested whether 4F3 was able to abolish the increase in platelet adhesion to collagen in the presence of apple4-C321S-β_2GPI. Whole anticoagulated blood was incubated for 5 min at 37°C with 100 µg/mL of apple4-C321S-β_2GPI and 100 µg/mL of 4F3 or 23H9. We used twice the amount of antibody compared to the other experiments since both β_2GPI in the blood and additional dimeric β_2GPI are recognized by 4F3. Afterwards, the blood was perfused for 90 s at a shear rate of 800 s^{-1} over collagen. As shown in fig. 5, in the presence of apple4-C321S-β_2GPI alone coverage increased to 148 ± 5 %, p<0.001), which is similar to the value shown in fig. 1. In the presence of both apple4-C321S-β_2GPI and 4F3, coverage decreased to 95 ± 9%, indicating that 4F3 is able to block the activation of platelets by dimeric β_2GPI. In contrast, monoclonal anti-β_2GPI antibody 23H9, which increases platelet adhesion to collagen, did not inhibit the increased deposition of platelets in the presence of apple4-C321S-β_2GPI (fig. 5).

To get more insight in about the receptor involved, we performed perfusion experiments in the presence of RAP, which interferes with the binding of ligands to members of the LDL-receptor family. As shown in fig. 6A, the increased platelet adhesion in the presence of apple4-β_2GPI (151 ± 19) decreased after preincubation with RAP to 108 ± 8% (p<0.01). RAP also abrogated the increased coverage observed in the presence of monoclonal anti-β_2GPI antibody 23H9 (141 ± 6% to 97 ± 24%, p<0.01; fig. 6B). To exclude the possibility that the Fcγ-receptor II on platelets is activated by the Fc-part of the anti-β_2GPI antibody, we performed an experiment in which whole anticoagulated blood was preincubated with monoclonal antibody IV.3, which blocks but does not activate the Fcγ-receptor II. As shown in fig. 6B, preincubation with antibody IV.3 did not affect the increased platelet coverage in the presence of anti-β_2GPI antibody 23H9 (134 ± 12), while the same antibody inhibited immune complex induced platelet aggregation (not shown).

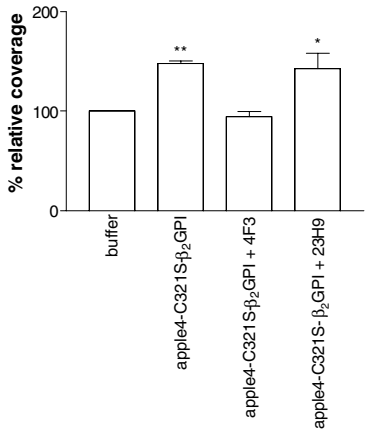

FIG. 5. **Effect of monoclonal anti-β_2GPI antibodies 4F3 and 23H9 on platelet deposition on collagen type III in the presence of apple4-C321S-β_2GPI.** Whole anticoagulated blood was incubated for 5 min at 37°C with apple4-C321S-β_2GPI (100 µg/mL) alone or with both apple4-C321S-β_2GPI and 4F3 or 23H9 (100 µg/mL) and perfused for 90 s at a shear rate of 800 s^{-1}. Platelet coverage was expressed as the percentage of surface covered with platelet relative to adhesion in the presence of buffer. Data are mean ± SD for 3 separate experiments. * p<0.05, ** p<0.001

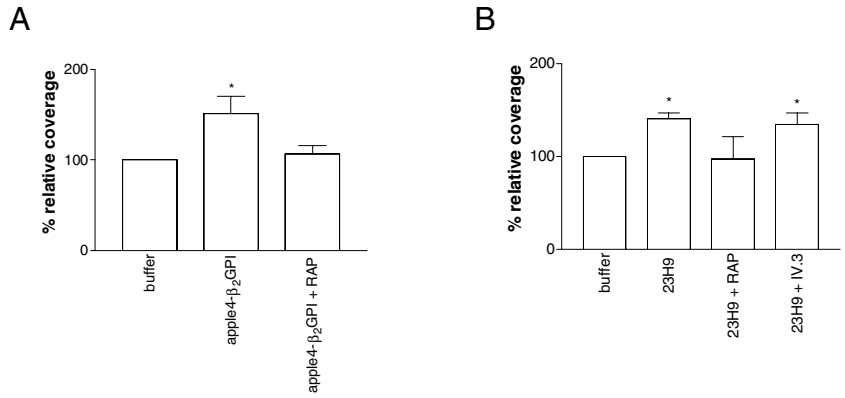

FIG. 6. **Effect of RAP and monoclonal antibody IV.3 on platelet coverage in the presence of apple4-β_2GPI or anti-β_2GPI antibody 23H9.** Whole anticoagulated blood was incubated for 5 min at 37ºC with RAP (10 µg/mL), followed by an incubation of 5 min at 37°C with apple4-β_2GPI (100 µg/mL)(*A*) or with 23H9 (50 µg/mL)(*B*) to determine the effect of RAP. The effect of Fc-receptor activation was measured by a 10 min incubation of whole blood with IV.3 (1 µg/mL) at 37°C, followed by an incubation of 5 min at 37°C with 23H9 (*B*). The blood was subsequently perfused for 90 s at a shear rate of 800 s^{-1}. Platelet coverage was expressed as the percentage of surface covered with platelet relative to adhesion in the presence of buffer. Data are mean ± SD for 3 separate experiments. * $p<0.01$

Discussion

The discrepancy between the presence of antibodies that prolong clotting assays in plasma and an increased risk for thrombo-embolic complications is still not understood. We and others have previously shown that dimerization of β_2GPI by antibodies is the cause of the prolongation of phospholipid-dependent clotting tests. We constructed chimeric dimers of β_2GPI that mimic the in vitro observed effects of anti-β_2GPI antibodies. Furthermore, we have shown that in contrast to dimeric β_2GPI, plasma-derived β_2GPI is not able to bind to platelets [36]. We now used the dimers of β_2GPI to investigate effects of dimer β_2GPI on platelet function. To do so, we perfused anticoagulated whole blood to which 100 µg/mL of dimeric β_2GPI was added over a collagen or fibronectin surface. The concentration we used is comparable to the concentration of β_2GPI in plasma (~200 µg/mL). Herewith, we showed that when fibronectin was used as a surface, platelet adhesion was 125% higher in the presence of apple4-β_2GPI or apple4-C321S-β_2GPI than in the presence of buffer, plasma-derived β_2GPI or the monomeric chimer. When collagen type III was used as a surface, platelet coverage in the presence of apple4-β_2GPI or apple4-C321S-β_2GPI increased with 50% compared to platelet coverage in the presence of buffer, plasma-derived β_2GPI, or the monomeric β_2GPI construct (fig. 1A). Further, it was shown that aggregates were larger in the presence of dimeric β_2GPI (fig. 2). These results indicate that dimerization of β_2GPI results in binding of β_2GPI to plate

lets. Platelet adhesion to fibrinogen did not increase in the presence of dimers of β_2GPI. A simple explanation for this is that upon binding to surface bound fibrinogen platelets do not expose negatively charged phospholipids, such as phosphatidylserine, on their membranes and that they do expose these negatively charged phospholipids after activation with collagen or fibronectin [42]. When no negatively charged phospholipids are exposed, the initial interaction of dimers of β_2GPI and β_2GPI-anti-β_2GPI antibody complexes cannot take place, and thus no effects on platelet adhesion are found. Our findings are contradictory to the findings of Ostfeld et al., who showed that platelet adhesion to collagen and fibronectin was decreased in the presence of LAC positive plasma of patients with systemic lupus erythematosus or purified IgG of these plasmas [43]. However, they used gel-filtered platelets and static adhesion experiments and we performed our experiments with whole anticoagulated blood under conditions of flow. Furthermore, we used antibodies against β_2GPI, but they did not characterize their antibodies. Thus, it is difficult to compare the results.

It has been shown that antiphospholipid antibodies can interfere with eicosanoid synthesis in platelets and endothelial cells [44-48]. TxA_2 is the main eicosanoid derived from platelets. It is rapidly inactivated into 11-dehydro-thromboxane B_2 via thromboxane B_2 and 2,3-dinor-thromboxane B_2. Released TxA_2 is able to activate platelets via the TxA_2 receptor, resulting in enhanced activation of the platelets. Antiphospholipid syndrome patients, with moderate or high titers of anti-β_2GPI antibodies, have higher levels of 11-dehydro-thromboxane B_2 in urine than controls [49]. Further, it has been shown that preincubation with β_2GPI-anticardiolipin antibody complexes results in production of higher levels of thromboxane B_2 by platelets [45]. Using inhibitors, we showed that thromboxane formation is essential in the activation of platelets by dimeric β_2GPI.

Using a panel of LAC positive anti-β_2GPI antibodies, we studied whether dimerization is a sine qua non to sensitize platelets. We have found that 2 out of 6 LAC positive monoclonal anti-β_2GPI antibodies had no effect on platelet adhesion. The LAC positive monoclonal antibodies 23H9, 27G7, 19H9, 22A11, and the purified patient antibodies MB and R showed an increased platelet adhesion to collagen. There are several explanations for the differences between induction of LAC activity and induction of increased platelet adhesion to collagen that we found for some LAC positive antibodies. First, the amount and composition of phospholipids used in the clotting assays is different from that present in the platelet membranes. Therefore, there may be discordance between the ability of the β_2GPI-anti-β_2GPI complex to prolong phospholipid-dependent coagulation assays and to adhere to platelets. To test this, we performed perfusions in the presence of anti-β_2GPI antibodies. Afterwards, binding of these antibodies to platelets was visualized by immunofluorescent labeling. As shown in table 2, all antibodies that induce LAC activity bound to platelets. Albeit that our immunofluorescent labeling experiments do not exclude quantitative differences, our findings suggest that lack of binding of β_2GPI-anti-β_2GPI antibody complexes to platelets is not a likely explanation for absence of platelet activating activity.

A better explanation for the different potential of LAC positive anti-β_2GPI antibodies to induce increased platelet deposition is that binding of dimerized β_2GPI to negatively charged phospholipids is not enough to sensitize platelets. Other proteins with high affinity for negatively charged phospholipids, such as annexin V, have no effect on platelet adhesion under flow [50]. The observation that a LAC positive antibody that did not induce increased

platelet adhesion, inhibited the increased platelet deposition induced by dimeric β_2GPI, indicates that after binding to negatively charged phospholipids, dimeric β_2GPI interacts with proteins on the platelet membrane. Apparently, after binding of dimeric β_2GPI to the platelet, an interaction of dimeric β_2GPI with a receptor on the platelet surface is involved in further activation. This receptor is clearly not the Fc-receptor, because the increase in adhesion observed in the presence of antibody 23H9 did not disappear after preincubation with monoclonal anti-Fcγ-receptor II antibody IV.3. Furthermore, Jankowski *et al.* reported recently that $F(ab)_2$ fragments of anti-β_2GPI antibody (5H2) were equally able to promote thrombus formation in a thrombosis model in the hamster [51]. This indicates that cellular activation via a Fc receptor is not the major receptor responsible for activation of platelets. Therefore a platelet receptor with specificity for β_2GPI may be involved. Possible candidates are members of the LDL-receptor family, since it has been described that β_2GPI can interact with megalin, a member of this family of endocytic receptors [52]. We showed that preincubation with RAP, which inhibits the interaction of ligands with members of the LDL-receptor family, abrogated increased adhesion in the presence of apple4-β_2GPI and anti-β_2GPI antibody 23H9. These results strongly suggest that a member of the LDL-receptor family is involved. The only member of the LDL-receptor family found on the platelet membrane, as far as known at present, is a splice variant of LRP8 (LRP8Δ4-6) [53]. However, to confirm the interaction of this receptor with the β_2GPI-anti-β_2GPI antibody complexes, experiments in which the binding of these complexes to the receptor is measured directly have to be performed.

Taken these observations together, we postulate a mechanism by which antiphospholipid antibodies can cause arterial thrombosis. In this, LAC positive β_2GPI-anti-β_2GPI antibody complexes bind to platelets via negatively charged phospholipids, which is followed by binding to a receptor, which is a member of the LDL-receptor family. Then dimerization of the receptor may occur, which results in the induction of signaling in which thromboxane formation plays a role. Finally, platelets become sensitized and are more prone to form a thrombus. This model might also fit for the activation of other cell types that become activated in the antiphospholipid syndrome as a lot of cells express members of the LDL-receptor family.

References

1. Kristensen T, Schousboe I, Boel E, Mulvihill EM, Hansen RR, Moller KB, Moller NPH, Sottrup-Jensen L. Molecular cloning and mammalian expression of human beta2-glycoprotein I cDNA. *FEBS Lett* 1991; 289: 183-186.
2. Steinkasserer A, Barlow PN, Willis AC, Kertesz Z, Campbell ID, Sim RB, Norman DG. Activity, disulphide mapping and structural modelling of the fifth domain of human beta 2-glycoprotein I. *FEBS Lett* 1992; 313: 193-197.
3. Lozier J, Takahashi N, Putnam FW. Complete amino acid sequence of human plasma beta 2-glycoprotein. *Proc Natl Acad Sci USA* 1984; 81: 3640-3644.
4. Kato H, Enjyoji K. Amino acid sequence and location of the disulfide bonds in bovine β2 glycoprotein I: The presence of five sushi domains. *Biochemistry* 1991; 30: 11687-11694.
5. Bendixen E, Halkier T, Magnusson S, Sottrup-Jensen L, Kristensen T. Complete primary structure of bovine β2-glycoprotein I: Localization of the disulfide bridges. *Biochemistry* 1992; 31: 3611-3617.
6. Hunt JE, Simpson RJ, Krilis SA. Identification of a region of β_2-glycoprotein I critical for lipid binding and anti-cardiolipin antibody cofactor activity. *Proc Natl Acad Sci USA* 1993; 90: 2141-2145.
7. Hunt J, Krilis S. The fifth domain of β_2-glycoprotein I contains a phospholipid binding site (Cys281-Cys288) and a region recognized by anticardiolipin antibodies. *J Immunol* 1994; 152: 653-659.
8. Sheng YH, Sali A, Herzog H, Lahnstein J, Krilis SA. Site-directed mutagenesis of recombinant human β_2-glycoprotein I identifies a cluster of lysine residues that are critical for phospholipid binding and anti-cardiolipin antibody activity. *J Immunol* 1996; 157: 3744-3751.
9. Mehdi H, Naqvi A, Kamboh MI. A hydrophobic sequence at position 313-316 (Leu-Ala-Phe-Trp) in the fifth domain of apolipoprotein H (β_2-glycoprotein I) is crucial for cardiolipin binding. *Eur J Biochem* 2000; 267: 1770-1776.
10. Horbach DA, Van Oort E, Tempelman MJ, Derksen RHWM, De Groot PhG. The prevalence of a non-phospholipid-binding form of β_2-glycoprotein I in human plasma. Consequences for the development of anti-β_2-glycoprotein I antibodies. *Thromb Haemost* 1998; 80: 791-797.
11. Bouma B, De Groot PhG, van den Elsen JMH, Ravelli RBG, Schouten A, Simmelink MJA, Derksen RHWM, Kroon J, Gros P. Adhesion mechanism of human β2-glycoprotein I to phospholipids based on its crystal structure. *EMBO J* 1999; 18: 5166-5174.
12. Schwartenbacher R, Zeth K, Diederichs K, Gries A, Kostner GM, Laggner P, Prassl R. Crystal structure of human β2-glycoprotein I: implication for phospholipid binding and the antiphospholipid syndrome. *EMBO J* 1999; 18: 6228-6239.
13. Schousboe I. β_2-glycoprotein I: A plasma inhibitor of the contact activation of the intrinsic blood coagulation pathway. *Blood* 1985; 66: 1086-1091.
14. Nimpf J, Bevers EM, Bomans PHH, Till U, Wurm H, Kostner GM, Zwaal RFA. Prothrombinase activity of human platelets is inhibited by beta2-glycoprotein-I. *Biochim Biophys Acta* 1986; 884: 142-149.

15. Shi W, Chong BH, Hogg PJ, Chesterman CN. Anticardiolipin antibodies block the inhibition by β_2-glycoprotein I of the factor Xa generating activity of platelets. *Thromb Haemost* 1993; 70: 342-345.
16. Keeling DM, Wilson AJG, Mackie IJ, Isenberg DA, Machin SJ. Role of β_2-glycoprotein I and anti-phospholipid antibodies in activation of protein C in vitro. *J Clin Pathol* 1993; 46: 908-911.
17. Matsuda J, Gohchi K, Kawasugi K, Gotoh M, Saitoh N, Tsukamoto M. Inhibitory activity of anti-β_2-glycoprotein I antibody on factor Va degradation by activated-protein C and its cofactor protein S. *Am J Hematol* 1995; 49: 89-91.
18. Schousboe I, Rasmussen MS. Synchronized inhibition of the phospholipid mediated autoactivation of factor XII in plasma by β_2-glycoprotein I and anti- β_2-glycoprotein I. *Thromb Haemost* 1995; 73: 798-804.
19. Mori T, Takeya H, Nishioka J, Gabazza EC, Suzuki K. β_2-Glycoprotein I modulates the anticoagulant activity of activated protein C on the phospholipid surface. *Thromb Haemost* 1996; 75: 49-55.
20. Ieko M, Yasukouchi T, Sawada K-I, Koike T. The influence of β2-glycoprotein I on tissue factor activity. *Semin Thromb Hemost* 1998; 24: 211-215.
21. Dahlback B, Nilsson IM, Frohm B. Inhibition of platelet prothrombinase activity by a lupus anticoagulant. *Blood* 1983; 62: 218-225.
22. Nimpf J, Wurm H, Kostner GM. Interaction of β_2-glycoprotein-I with human blood platelets: influence upon the ADP-induced aggregation. *Thromb Haemost* 1985; 54: 397-401.
23. Schousboe I. Binding of beta 2-glycoprotein I to platelets: effect of adenylate cyclase activity. *Thromb Res* 1980; 19: 225-237.
24. Chonn A, Semple SC, Cullis PR. β 2-Glycoprotein I is a major protein associated with very rapidly cleared liposomes in vivo, suggesting a significant role in the immune clearance of «non-self»particles. *J Biol Chem* 1995; 270: 25845-25849.
25. Wurm H. Beta 2-glycoprotein-I (apolipoprotein H) interactions with phospholipid vesicles. *Int J Biochem* 1984; 16: 511-515.
26. Hasunuma Y, Matsuura E, Makita Z, Katahira T, Nishi S, Koike T. Involvement of β_2-glycoprotein I and anticardiolipin antibodies in oxidatively modified low-density lipoprotein uptake by macrophages. *Clin Exp Immunol* 1997; 107: 569-573.
27. Balasubramanian K, Chandra J, Schroit A. Immune clearance of phosphatidylserine-expressing cells by phagocytes. The role of β2-glycoprotein I in macrophage recognition. *J Biol Chem* 1997; 272: 31113-31117.
28. Oosting JD, Derksen RHWM, Entjes HTI, Bouma BN, De Groot PhG. Lupus anticoagulant activity is frequently dependent on the presence of β_2-glycoprotein I. *Thromb Haemost* 1992; 67: 499-502.
29. Roubey RAS, Pratt CW, Buyon JP, Winfield JB. Lupus anticoagulant activity of auto-immune antiphospholipid antibodies is dependent upon beta 2-glycoprotein I. *J Clin Invest* 1992; 90: 1100-1104.
30. Love PE, Santoro SA. Antiphospholipid antibodies: anticardiolipin and the lupus anticoagulant in systemic lupus erythematosus (SLE) and in non-SLE disorders. Prevalence and clinical significance. *Ann Intern Med* 1990; 112: 682-698.

31. Roubey RAS. Autoantibodies to phospholipid-binding plasma proteins: a new view of lupus anticoagulants and other "antiphospholipid" autoantibodies. *Blood* 1994; 84: 2854-2867.
32. Harris EN, Asherson RA, Hughes GR. Antiphospholipid antibodies-autoantibodies with a difference. *Annu Rev Med* 1988; 39: 261-271.
33. De Groot PhG, Oosting JD, Derksen RHWM. Antiphospholipid antibodies: specificity and pathophysiology. *Bailliere's Clin Haematol* 1993; 6: 691-709.
34. Horbach DA, Van Oort E, Donders RCJM, Derksen RHWM, De Groot PG. Lupus anticoagulant is the strongest risk factor for both venous and arterial thrombosis in patients with systemic lupus erythematosus - Comparison between different assays for the detection of antiphospholipid antibodies. *Thromb Haemost* 1996; 76: 916-924.
35. Wilson WA, Gharavi AE, Koike T, Lockshin MD, Branch DW, Piette JC, Brey R, Derksen RHWM, Harris EN, Hughes GRV, Triplett DA, Khamashta MA. International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: report of an international workshop. *Arthritis Rheum* 1999; 42: 1309-1311.
36. Lutters BCH, Meijers JCM, Derksen RHWM, Arnout J, De Groot PG. Dimers of β_2-glycoprotein I mimic the in vitro effects of β_2-glycoprotein I-anti-β_2-glycoprotein I antibody complexes. *J Biol Chem* 2001; 276: 3060-3067.
37. Houdijk WPM, Sakariassen KS, Nievelstein PFEM, Sixma JJ. Role of factor VIII-von Willebrand Factor and fibronectin in the interaction of platelets in flowing blood with monomeric and fibrillar collagen types I and III. *J Clin Invest* 1985; 75: 531-540.
38. Lenting PJ, Neels JG, van den Berg BMM, Clijsters PPFM, Meijerman DWE, Pannekoek H, Van Mourik JA, Mertens K, Van Zonneveld A-J. The light chain of factor VIII comprises a binding site for low density lipoprotein receptor-related protein. *J Biol Chem* 1999; 274: 23734-23739.
39. Arnout J, Wittevrongel C, Vanrusselt M, Hoylaerts M, Vermylen J. Beta-2-glycoprotein I dependent lupus anticoagulants form stable bivalent antibody beta-2-glycoprotein I complexes on phospholipid surfaces. *Thromb Haemost* 1998; 79: 79-86.
40. van Zanten HG, Saelman EUM, Schut Hese KM, Wu YP, Slootweg PJ, Nieuwenhuis HK, De Groot PG, Sixma JJ. Platelet adhesion to collagen type IV under flow conditions. *Blood* 1996; 88: 3862-3871.
41. Sixma JJ, De Groot PG. Regulation of platelet adhesion to the vessel wall. *Ann N Y Acad Sci* 1994; 714: 190-199.
42. Heemskerk JWM, Vuist WMJ, Feijge MAH, Reutelingsperger CPM, Lindhout T. Collagen but not fibrinogen surfaces induce bleb formation, exposure of phosphatidylserine, and procoagulant activity of adherent platelets: evidence for regulation by protein tyrosine kinase-dependent Ca^{2+} responses. *Blood* 1997; 90: 2615-2625.
43. Ostfeld I, Dadosh-Goffer N, Borokowski S, Talmon J, Mani A, Zor U, Lahav J. Lupus anticoagulant antibodies inhibit collagen-induced adhesion and aggregation of human platelets in vitro. *J Clin Immuno*l 1992; 12: 415-423.
44. Watson KV, Schorer AE. Lupus anticoagulant inhibition of in vitro prostacyclin release is associated with a thrombosis-prone subset of patients. *Am J Med* 1991; 90: 47-53.
45. Robbins DL, Leung S, Miller-Blair DJ, Ziboh VA. Effect of anticardiolipin/β2-glycoprotein

I complexes on production of thromboxane A2 by platelets from patients with the antiphospholipid syndrome. *J Rheumatol* 1998; 25: 51-56.
46. Martinuzzo M, Maclouf J, Carreras LO, Lé vy-Toledano S. Antiphospholipid antibodies enhance thrombin-induced platelet activation and thromboxane formation. *Thromb Haemost* 1993; 70: 667-671.
47. Carreras LO, Machin SJ, Deman R, Defreyn G, Vermylen J, Spitz B, Van Assche A. Arterial thrombosis, intrauterine death and "lupus" anticoagulant: detection of immunoglobulin interfering with prostacyclin formation. *Lancet* 1981; 1: 244-246.
48. Schorer AE, Wickham NWR, Watson KV. Lupus anticoagulant induces a selective defect in thrombin-mediated endothelial prostacyclin release and platelet aggregation. *Br J Haematol* 1989; 71: 399-407.
49. Forastiero RR, Martinuzzo M, Carreras LO, Maclouf J. Anti-β_2-glycoprotein I antibodies and platelet activation in patients with antiphospholipid syndrome: association with increased excretion of platelet-derived thromboxane urinary metabolites. *Thromb Haemost* 1998; 79: 42-45.
50. Van Heerde WL, Sakariassen KS, Hemker HC, Sixma JJ, De Groot PhG. Annexin V inhibits the procoagulant activity of matrices of TNF-stimulated endothelium under blood flow conditions. *Arterioscler Thromb* 1994; 14: 824-830.
51. Jankowski M, Vreys I, Wittevrongel C, Boon D, Vermylen J, Hoylaerts MF, Arnout J. Thrombogenecity of beta2-glycoprotein I dependent antiphospholipid antibodies in a photochemically induced thrombosis model in the hamster. *Blood* 2002 [epub ahead of print].
52. Moestrup SK, Schousboe I, Jacobsen C, Leheste JR, Christensen EI, Willnow TE. β_2-glycoprotein I (apolipoprotein H) and β_2-glycoprotein I-phospholipid complex harbor a recognition site for the endocytic receptor megalin. *J Clin Invest* 1998; 102: 902-909.
53. Riddell DR, Vinogradov DV, Stannard AK, Chadwick N, Owen JS. Identification and characterization of LRP8 (apoER2) in human blood platelets. *J Lip Res* 1999; 40: 1925-1930.

Chapter 5

Comparison of the effects of anti-β_2-glycoprotein I and anti-prothrombin antibodies on platelet deposition in an in vitro flow system

Bianca C.H. Lutters[1,3], Ronald H.W.M. Derksen[2], Winnie L.Tekelenburg[1], Jef Arnout[4], Philip G. de Groot[1,3]

Departments of [1]Haematology and [2]Rheumatology and Clinical Immunology, University Medical Center, Utrecht and [3]Institute of Biomembranes, Utrecht University, [4]Center for Molecular and Vascular Biology, University of Leuven, Leuven, Belgium.

Abstract

It is now widely accepted that most antiphospholipid antibodies are not directed against phospholipid directly, but against phospholipid-bound proteins, like β_2-glycoprotein I (β_2GPI) and prothrombin. To compare the effects of anti-β_2GPI and anti-prothrombin antibodies on platelet reactivity, we have used an *in vitro* flow system in which whole anticoagulated blood was perfused over collagen in the presence of anti-β_2GPI or anti-prothrombin antibodies (50 µg/mL). We found that preincubation with 5 out of 7 monoclonal anti-β_2GPI antibodies or two purified patient anti-β_2GPI antibodies, all being lupus anticoagulant (LAC) positive resulted in increased platelet adhesion to collagen. The LAC negative anti-β_2GPI antibodies 1F12 and KJ had no effect on platelet adhesion. None of the LAC positive anti-prothrombin antibodies (monoclonal, polyclonal and purified patient) had an effect on platelet adhesion to collagen. We also tested binding of the antibodies to platelets after perfusion over collagen using immunofluoresence. We showed that all LAC positive anti-β_2GPI antibodies were able to bind to platelets. No binding was detected for the LAC negative anti-β_2GPI antibody and for the anti-prothrombin antibodies. This indicates that LAC positive anti-β_2GPI antibodies, but not anti-prothrombin antibodies, can sensitize platelets, resulting in increased deposition of platelets on collagen. Moreover, the results suggest that binding of antiphospholipid antibodies to platelets is necessary to exert effects on platelet activation, although binding to by itself is not enough to increase in platelet adhesion, as was shown by the anti-β_2GPI antibodies 4F3 and 2B2.

Introduction

The presence of antiphospholipid antibodies in plasma is associated with a risk for thrombotic complications. This association is called the antiphospholipid syndrome [1-6]. It is now generally accepted that these antiphospholipid antibodies are not directed against phospholipids directly, but against phospholipid-bound proteins, of which β_2-glycoprotein I (β_2GPI) [7-9] and prothrombin [10] are the most important ones. Antibodies against β_2GPI [11,12] and prothrombin [10] both can cause prolongation of phospholipid-dependent coagulation assays, known as lupus anticoagulant (LAC) activity. LAC activity has been proven to be an independent risk factor for the development of thrombosis [5,13-15].

Nowadays, it is not clear whether anti-β_2GPI or anti-prothrombin antibodies are more important for the development of thrombosis. Palusuo *et al.* performed a case-control study, in which they found that the presence of anti-prothrombin, and not anti-β_2GPI, antibodies was associated with the occurrence of venous thrombosis [16]. Horbach *et al.* [5] and Forastiero *et al.* [17] found in their retrospective studies for both the presence of anti-prothrombin and anti-β_2GPI antibodies an association with venous thrombosis. Horbach *et al.* showed also a correlation between the presence of anti-β_2GPI antibodies of the IgM isotype and a history of arterial thrombosis [5], although with multilogistic regression analysis the presence of none of these antibodies was an independent risk factor for arterial or venous thrombosis in this study. In contrast, the presence of anti-β_2GPI antibodies of the IgG isotype were an independent risk factor for arterial thrombosis in another study [17]. In a case-control study, Galli *et al.* found that the presence of anti-prothrombin antibodies, measured with both prothrombin alone and prothrombin bound to phosphatidylserine as antigen, was not correlated with thrombosis [18]. Also, no correlation between the presence of anti-prothrombin antibodies and thrombosis was found by Pengo *et al.* [19], although they did find a correlation between the presence of anti-β_2GPI antibodies and the occurrence of thrombosis.

To investigate the effects of anti-β_2GPI and anti-prothrombin antibodies on platelet function, we use an *in vitro* flow system in which whole anticoagulated blood was perfused over collagen. We conclude that several LAC positive anti-β_2GPI, but not anti-prothrombin or LAC negative anti-β_2GPI antibodies, bind to and sensitize platelets, resulting in increased platelet adhesion to collagen.

Materials and methods

Antibodies

Patient anti-β_2GPI (MB, R and KJ) and anti-prothrombin (BP and LB) antibodies were purified from plasma using β_2GPI or prothrombin coupled to CNBr-activated Sepharose, respectively (Amersham Pharmacia Biotech, Uppsala, Sweden). Bound antibodies were eluted with 3 M KCNS in tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4) and dialysed against TBS. Monoclonal anti-β_2GPI antibody 4F3 was purified using a protein G column. Bound antibodies were eluted with 0.1 M glycine, pH 2.7, neutralised with 1 M Tris, pH 9, and dialysed against TBS. Protein concentration was determined using a bichinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) according to the instructions of the

manufacturer, and with bovine serum albumin (BSA) as a standard. Monoclonal anti-β_2GPI antibodies 22A11, 23H9, 27G7, and 19H9 and monoclonal anti-prothrombin antibody 28F4 were described previously [20,21]. Polyclonal rabbit-anti-human anti-prothrombin antibody (anti-FII (PoAb)) was obtained from DAKO (Glostrup, denmark). Monoclonal anti-β_2GPI antibodies 2B2 and 1F12 were kindly provided by Dr. A. Tincani (Clinical Immunology Unit, Spedali Civili, Brescia, Italy).

Patients

Anti-β_2GPI antibodies were isolated from plasma from five female patients with antiphospholipid syndrome (MB, KJ and R, with informed consent). Two of these anti-β_2GPI antibodies were LAC positive after isolation (MB and R) and one antibody was LAC negative (KJ; see determination of LAC activity). Patient MB (age 32) was LAC positive and had IgG class anti-β_2GPI antibodies [5]. She had mild, chronic thrombocytopenia, cerebral infarction and recurrent first and second trimester pregnancy loss as manifestations of the antiphospholipid syndrome. Patient KJ (44 years) had lupus like disease and thrombosis of the distal aorta and patient R (age 46) had primary antiphospholipid syndrome (multiple cerebral infarctions, chronic mild thrombocytopenia and livedo reticularis). Plasma from both patient KJ and R were LAC positive and contained anti-β_2GPI (IgG isotype) and anti-prothrombin antibodies (IgG and IgM isotype). Plasma from patient R was also positive for IgM class anti-β_2GPI antibodies.

Anti-prothrombin antibodies were purified from plasma from two LAC positive antiphospholipid syndrome patients (BP and LB, with informed consent). Patient BP (age 30) suffered from recurrent venous (deep vein thrombosis and pulmonary emboli) and arterial (transient ischaemic attacks and ischaemic stroke) thrombosis and first trimester pregnancy loss. Her plasma was positive for anti-prothrombin (IgG isotype) antibodies. Patient LB (age 39) had pulmonary emboli, livedo reticularis and Coombs' positive haemolytic anaemia as manifestations of the antiphospholipid syndrome. Her plasma was positive for anti-prothrombin (IgG and IgM isotype) and for anti-β_2GPI (IgG and IgM isotype) antibodies.

Surfaces

Human placental collagen type III (Sigma, St Louis, MO, USA) was solubilized overnight in 50 mmol/L acetic acid and sprayed onto Thermanox^R coverslips at a density 30 µg/mL with a retouching airbrush (model 100, Badger Brush Co). For the immunofluoresence studies glass coverslips (18 x 18 mm; Menzel Glä ser, Braunschweig, Germany) were used. After spraying, the coverslips were blocked for at least 30 minutes at room temperature with 1% human serum albumin (ICN Biomedicals, Inc., Aurora, OH, USA) in phosphate-buffered saline, pH 7.4 (PBS).

Blood collection

Freshly drawn venous blood was collected from healthy donors (with informed consent), who did not use aspirin or other platelet function inhibitors during the last ten days, into 1/10 of volume of 3.2% tri-sodium citrate (w/v) or into 1/10 of volume of low molecular weight heparin (LMWH, 200 U/mL; Fragmin®, Pharmacia, Woerden, The Netherlands). For the studies with anti-β_2GPI antibodies blood anticoagulated with citrate

was used. Anti-prothrombin antibodies were added to blood anticoagulated with LMWH. These different anticoagulants were used since β_2GPI can interact with heparin and the binding of prothrombin to negatively charged phospholipids is dependent on the presence of calcium ions.

Perfusate

5 min prior to the perfusion, anti-β_2GPI and anti-prothrombin antibodies were added at a concentration of 50 µg/mL to whole blood and incubated at 37°C. As a control, buffer (TBS) was added to whole blood.

Perfusion studies

Perfusion were performed in a single-pass perfusion chamber under nonpulsatile flow conditions using a modified parallel plate perfusion with a slit width of 2 mm and a slit height of 0.1 mm [22]. Experiments with collagen type III as a surface were done with a perfusion time of 90 s, at a shear rate of 800 s^{-1}, which corresponds to the shear rate in arterioles. The prewarmed blood was drawn through the perfusion chamber by an infusion pump (pump 22, model 2400-004; Harvard, Natrick, MA, USA). Afterwards, the coverslips were taken from the perfusion chamber and rinsed with HEPES-buffered saline (10 mmol/L HEPES, 150 mmol/L NaCl, pH 7.4), fixed in 0.5% glutaraldehyde in PBS, dehydrated with methanol and stained with May-Grünwald/Giemsa as previously described [23]. Platelet deposition was evaluated with a light microscope equipped with a JAI-CCD camera (Copenhagen, Denmark) coupled to a Matrox frame grabber (Matrox Electronic Systems Ltd., Quebec, Canada) using Optimas 6.2 software (Optimas Inc, Seattle, WA, USA) for image analysis. Evaluation was performed on thirty fields, perpendicular to the flow direction. Platelet adhesion was expressed as the percentage of the surface covered with platelets relative to adhesion in the presence of buffer.

Determination of LAC activity

Coagulation assays were performed in a KC-10 coagulometer (Amelung, Lemgo, Germany). To detect LAC activity, three different coagulation assays were used: an activated partial thromboplastin time-based assay sensitized to detect LAC, (PTT-LA; Diagnostica Stago, Asnieres-sur-Seine, France), a tissue factor-based assay using innovin (Dade Behring, Marburg, Germany), and a dilute Russell's viper venom time (dRVVT; Gradipore Ltd, North Ryde, Australia). All were performed as described previously [21]. Anti-β_2GPI and anti-prothrombin antibodies were added at a concentration of 100 µg/mL to the plasma, which is similar to a concentration of 50 µg/mL in whole blood as used in the perfusions. Clotting time ratios were calculated by dividing the clotting time in the presence of antibody by the clotting time in the absence of antibody. Ratios ≥ 1.2 were considered to be positive. An anti-β_2GPI or anti-prothrombin antibody is LAC positive when one of the coagulation assays performed was positive. As shown in table 1, monoclonal anti-β_2GPI antibody 22A11 and monoclonal anti-prothrombin antibody 28F4 were LAC positive in all three coagulation assays. The monoclonal anti-β_2GPI antibody 27G7 was positive with the tissue factor-based assay and the dRVVT and the anti-FII (PoAb) antibody was positive in both the PTT-LA and the dRVVT. The other monoclonal anti-β_2GPI antibodies (23H9 and 19H9) were LAC positive in

the tissue factor-based assay only. The monoclonal anti-β_2GPI antibody (1F12) was negative in all three assays. The purified patient anti-β_2GPI antibodies (MB and R) were positive in the PTT-LA and dRVVT, respectively. The purified patient anti-β_2GPI antibody KJ was negative. One of the purified patient anti-prothrombin antibodies (LB) was positive in the dRVVT and another (BP) was positive in both the dRVVT, and the PTT-LA. When 100 µg/mL of antibody BP was added to the PTT-LA, the clotting time ratio was more than 10. With 12.5 µg/mL this antibody had a clotting time ratio of 3.13.

TABLE 1. **Effect of anti-β_2GPI and anti-prothrombin antibodies on coagulation assays**. Anti-β_2GPI or anti-prothrombin antibodies were added at a concentration of 100 µg/mL to pooled normal plasma. Afterwards a tissue factor-based assay (innovin), a PTT-LA, and a dRVVT were performed. The clotting time in the presence of antibody was divided by the clotting time in the absence of an antibody. Ratios ≥ 1.2 were considered to be positive (#).

Antibody	Innovin	PTT-LA	dRVVT	LAC
MB	1.07	1.53#	1.04	+
R	1.06	1.00	1.72#	+
23H9	1.88#	1.19	1.11	+
27G7	1.32#	1.16	1.56#	+
19H9	1.29#	1.19	1.10	+
22A11	1.71#	1.33#	1.26#	+
2B2	1.56#	1.43#	1.45#	+
4F3	1.50#	1.19	1.12	+
KJ	1.04	1.06	1.00	-
1F12	1.00	1.16	1.11	-
BP	1.10	1.56#	>10#*	+
LB	1.05	1.15	1.65#	+
28F4	1.94#	1.86#	1.74#	+
Anti-FII (PoAb)	1.09	1.20#	1.43#	+

* In the presence of 12.5 µg/mL of antibody, the ratio was 3.13.

Immunofluoresence studies

Whole anticoagulated blood was perfused over collagen type III in the presence of different anti-β_2GPI and anti-prothrombin antibodies. Binding of these antibodies to platelets was detected using immunofluorescent labelling of these antibodies. After perfusion coverslips were fixed with 3% paraformaldehyde/0.005% glutardialdehyde in PBS, pH 7.4, washed three times with PBS, pH 7.4, and blocked for 10 min with PBS containing 1% BSA and 0.1% glycine, pH 7.4. Afterwards coverslips were incubated with a fluorescein isothiocyanate (FITC)-labeled goat-anti-mouse antibody (Becton Dickinson, San Jose, CA, USA), diluted

1:20 in PBS/1% BSA/0.1% glycine, pH 7.4 for 45 min at 37ºC. The polyclonal anti-prothrombin antibody was detected with a tetramethyl rhodamine isothiocyanate (TRITC)-labeled goat-anti-rabbit antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). This was followed by three times washing with PBS. For detection of anti-prothrombin antibodies, PBS was replaced by TBS, and all incubations were performed in the presence of 3 mM of $CaCl_2$. Finally, coverslips were mounted in MOWIOL 4-88 (Calbiochem, La Jolla, CA, USA) and 0.1% PPD (para-phenylene diamine, Sigma, St Louis, MO, USA).

Statistical analysis

Perfusions were performed three times in duplicate for each condition. The results are presented as mean ± standard deviation (SD) from experiments with blood from three different healthy donors (N=3). The difference between the various conditions was tested by analysis of variance (ANOVA) and Bonferoni was used as post-test. The results were considered significant at $P<0.05$.

Results

Effects of anti-β_2GPI and anti-prothrombin antibodies on platelet adhesion to collagen

To investigate the differences in thrombotic potential between LAC positive anti-β_2GPI and anti-prothrombin antibodies, we have determined the effects of these antibodies on platelet adhesion to collagen in an *in vitro* flow system. To do so, we have incubated whole anticoagulated blood with 50 µg/mL of anti-β_2GPI or anti-prothrombin antibodies. This concentration of antibody is similar to the 100 µg/mL of antibody that was added to plasma to determine the potential of the antibodies to induce LAC activity. Subsequently, the blood was perfused over a collagen surface at a shear rate of 800 s^{-1}. The coverage in the presence of buffer was set at 100%. As shown in fig. 1A, four monoclonal LAC positive anti-β_2GPI antibodies, namely 23H9 (157 ± 13%, $p<0.01$), 27G7 (156 ± 28%, $p<0.01$), 19H9 (180 ± 7%, $p<0.001$), and 22A11 (132 ± 14%, $p<0.01$) induced a significant increase in platelet adhesion to collagen. The LAC positive patient anti-β_2GPI antibodies MB (135 ± 9%, $p<0.01$) and R (133 ± 10 %, $p<0.01$) also induced an increase in platelet coverage. Preincubation with the LAC positive anti-β_2GPI antibodies 2B2 (99 ± 3%) and 4F3 (107 ± 4%) and the LAC negative anti-β_2GPI antibodies 1F12 (113 ± 18%) and KJ (101 ± 5%) did not influence platelet adhesion. Also, preincubation with the monoclonal anti-prothrombin antibody 28F4 (100 ± 1%), the anti-FII (PoAb) antibody (100 ± 2%), and the patient anti-prothrombin antibodies BP (100 ± 4%) and LB (98 ± 1%) did not affect platelet adhesion to collagen (fig. 1B).

Binding of anti-β_2GPI and anti-prothrombin antibodies to platelets

Since preincubation with LAC positive anti-prothrombin antibodies, in contrast to LAC positive anti-β_2GPI antibodies, did not result in an increased platelet adhesion to collagen under flow, we determined whether anti-β_2GPI and anti-prothrombin antibodies could be detected on the platelet surface after perfusion by immunofluoresence. As shown in table 2, all LAC positive monoclonal anti-β_2GPI antibodies bound to platelets, whereas the LAC negative anti-β_2GPI antibody and monoclonal and polyclonal anti-prothrombin antibodies

did not bind to platelets.

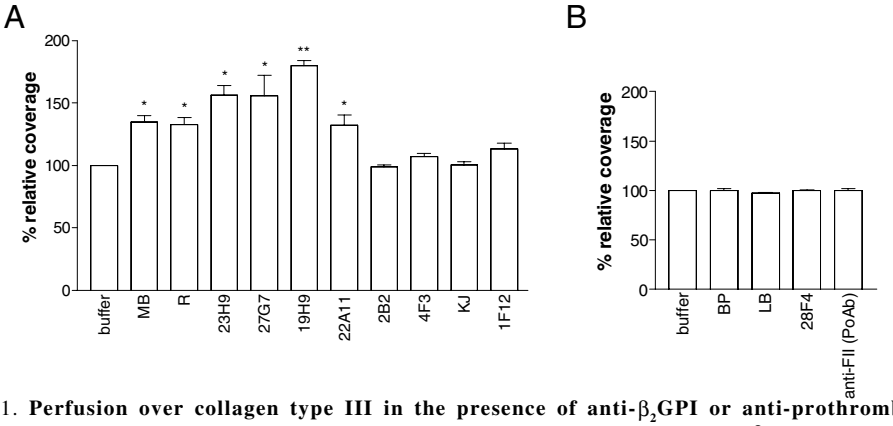

FIG.1. **Perfusion over collagen type III in the presence of anti-β_2GPI or anti-prothrombin antibodies.** Whole anticoagulated blood was incubated for 5 min at 37-C with anti-β_2GPI (*A*) or anti-prothrombin antibodies (*B*) (50 ®g/mL). Blood was perfused at a shear rate of 800 s^{-1} for 90 s. Platelet adhesion was expressed as the percentage of the surface covered with platelets relative to adhesion in the presence of buffer. Data are mean ± SD for three separate experiments. * $p<0.01$, ** $p<0.001$

Antibody	Binding to platelets
23H9	+
27G7	+
19H9	+
22A11	+
2B2	+
4F3	+
1F12	-
28F4	-
Anti-FII (PoAb)	-

TABLE 2. **Binding of anti-β_2GPI or anti-prothrombin antibodies to platelets in an *in vitro* perfusion system.** Whole anticoagulated blood was incubated 5 min at 37ºC with 50 ®g/ml of antibody and afterwards perfused for 90 s over a collagen surface. Binding of the antibodies was detected with immunofluoresence.

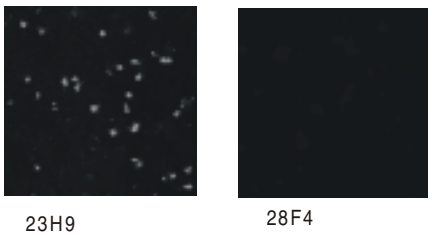

FIG.2. **Binding of anti-β_2GPI or anti-prothrombin antibodies to platelets adhered to collagen.** Whole anticoagulated blood was incubated for 5 min at 37ºC with anti-β_2GPI (23H9) or anti-prothrombin anttibodies (28F4). Blood was perfused for 90 s at a shear rate of 800 s^{-1}. Binding of antibodies to adhered platelets was visualized using immunofluoresence.

Discussion

It is now generally accepted that antiphospholipid antibodies are not directed against phospholipids directly, but against phospholipid-bound proteins, of which β_2GPI and prothrombin are reported to be most important *in vivo. In vitro*, it has been reported that anti-β_2GPI antibodies are thrombogenic, as shown by activation of endothelial cells and platelets [24-26]. However, little is known about the effects of anti-prothrombin antibodies on these cell-types. Of one monoclonal anti-prothrombin antibody it has been described that it enhances the binding of prothrombin to endothelial cells [27]. To further investigate the effects of anti-prothrombin antibodies, and to compare these effects with those of anti-β_2GPI antibodies, we incubated whole anticoagulated blood with LAC positive anti-β_2GPI or anti-prothrombin antibodies. Blood spiked with these antibodies was subsequently perfused over a collagen surface at a shear rate of 800 s^{-1}, which is representative for the shear rate in small arteries.

We showed that preincubation with five out of seven LAC positive anti-β_2GPI antibodies resulted in an increase in platelet adhesion to collagen. The two other LAC positive anti-β_2GPI antibodies, the LAC negative anti-β_2GPI antibody, and all four LAC positive anti-prothrombin antibodies did not affect platelet adhesion to collagen (fig. 1). Furthermore, all LAC positive anti-β_2GPI antibodies did bind to platelets, in contrast to the LAC negative anti-β_2GPI antibody and all anti-prothrombin antibodies, which did not bind (table 2). For anti-β_2GPI antibodies, we previously suggested a mechanism by which they increase the adhesion of platelets to a thrombogenic surface [28]. In short, the platelets become slightly activated by the thrombogenic surface (e.g. collagen) and express negatively charged phospholipids on their surface. Subsequently, β_2GPI-anti-β_2GPI antibody complexes bind to the exposed negatively charged phospholipids. This is followed by the interaction of β_2GPI-anti-β_2GPI antibody complexes with a receptor specific for β_2GPI on the platelet surface. Then dimerization of this receptor may occur, which results in the induction of signal transduction and thus further activation of platelets. The results obtained with the anti-prothrombin antibodies show that the mechanism of action described for anti-β_2GPI does not apply for and anti-prothrombin antibodies, probably simply because they do not bind to the adhered platelets.

Our data can be interpreted as that anti-prothrombin antibodies are not the pathogenic antibodies for arterial complications in the antiphospholipid syndrome. This is supported by the fact that some clinical studies reported an association between the presence of these antibodies in the circulation and thrombosis [5,16,17]. Furthermore, previous studies demonstrated that anti-prothrombin antibodies can interact with endothelial cells [27], but about their ability to interact with platelets nothing is known at present. Also, it has never been studied whether anti-prothrombin antibodies can activate endothelial cells or platelets. Besides the activation of cells, anti-prothrombin antibodies might have a procoagulant effect on the coagulation cascade by inhibition of the activation of protein C to activated protein C (APC), resulting in a prothrombotic state via a decreased inhibition of the coagulation cascade. Indeed, Simmelink *et al.* did find that low levels of APC were related to the presence of anti-prothrombin antibodies, although these low levels of APC were not associated with thrombosis [29].

In summary, our data show that LAC positive anti-β_2GPI antibodies, and not LAC positive anti-prothrombin antibodies, are able to sensitize platelets, which results in an increased deposition of the platelets on collagen in an *in vitro* flow system. Further research

on the effects of anti-prothrombin antibodies on coagulation and the activation of endothelial cells and platelets is needed to determine mechanisms by which these antibodies exert their *in vivo* effects.

References

1. Love PE, Santoro SA. Antiphospholipid antibodies: anticardiolipin and the lupus anticoagulant in systemic lupus erythematosus (SLE) and in non-SLE disorders. Prevalence and clinical significance. *Ann Intern Med* 1990; 112: 682-698.
2. Roubey RAS. Autoantibodies to phospholipid-binding plasma proteins: a new view of lupus anticoagulants and other "antiphospholipid" autoantibodies. *Blood* 1994; 84: 2854-2867.
3. Harris EN, Asherson RA, Hughes GR. Antiphospholipid antibodies-autoantibodies with a difference. *Annu Rev Med* 1988; 39: 261-271.
4. De Groot PhG, Oosting JD, Derksen RHWM. Antiphospholipid antibodies: specificity and pathophysiology. *Bailliere's Clin Haematol* 1993; 6: 691-709.
5. Horbach DA, Van Oort E, Donders RCJM, Derksen RHWM, De Groot PG. Lupus anticoagulant is the strongest risk factor for both venous and arterial thrombosis in patients with systemic lupus erythematosus - Comparison between different assays for the detection of antiphospholipid antibodies. *Thromb Haemost* 1996; 76: 916-924.
6. Wilson WA, Gharavi AE, Koike T, Lockshin MD, Branch DW, Piette JC, Brey R, Derksen RHWM, Harris EN, Hughes GRV, Triplett DA, Khamashta MA. International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: report of an international workshop. *Arthritis Rheum* 1999; 42: 1309-1311.
7. Galli M, Comfurius P, Maassen C, Hemker HC, de Baets MH, van Breda-Vriesman PJC, Barbui T, Zwaal RFA, Bevers EM. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein factor. *Lancet* 1990; 335: 1544-1547.
8. McNeil HP, Simpson RJ, Chesterman CN, Krilis SA. Antiphospholipid antibodies are directed against a complex antigen that includes a lipid binding inhibitor of coagulation: β_2-glycoprotein I (apolipoprotein H). *Proc Natl Acad Sci USA* 1990; 87: 4120-4124.
9. Matsuura E, Igarashi Y, Fujimoto M, Ichikawa K, Koike T. Anticardiolipin cofactor(s) and differential diagnosis of autoimmune disease. *Lancet* 1990; 336: 177-178.
10. Bevers EM, Galli M, Barbui T, Comfurius P, Zwaal RFA. Lupus anticoagulant IgG's (LA) are not directed to phospholipids only, but to a complex of lipid-bound human prothrombin. *Thromb Haemost* 1991; 66: 629-632.
11. Oosting JD, Derksen RHWM, Entjes HTI, Bouma BN, De Groot PhG. Lupus anticoagulant activity is frequently dependent on the presence of β_2-glycoprotein I. *Thromb Haemost* 1992; 67: 499-502.
12. Roubey RAS, Pratt CW, Buyon JP, Winfield JB. Lupus anticoagulant activity of autoimmune antiphospholipid antibodies is dependent upon beta 2-glycoprotein I. *J Clin Invest* 1992; 90: 1100-1104.
13. D'Angelo A, Safa O, Crippa L, Garlando A, Sabbadini MG, D'Angelo SV. Relationship of lupus anticoagulant, anticardiolipin, anti-β_2-GPI and anti-prothrombin autoantibodies with history of thrombosis in patients with the clinical suspicion of APA-syndrome. *Thromb Haemost* 1997; 78: 967-968.
14. Wahl DG, Guillemin F, de Maistre E, Perret C, Lecompte T, Thibaut G. Risk for venous thrombosis related to antiphospholipid antibodies in systemic lupus erythematosus-a meta-analysis. *Lupus* 1997; 6: 467-473.

15. Wahl DG, Guillemin F, de Maistre E, Perret-Guillaume C, Lecompte T, Thibaut G. Meta-analysis of the risk of venous thrombosis in individuals with antiphospholipid antibodies without underlying autoimmune disease or previous thrombosis. *Lupus* 1998; 7: 15-22.
16. Palosuo T, Virtamo J, Haukka J, Taylor PR, Aho K, Puurunen M, Vaarala O. High antibody levels to prothrombin imply a risk of deep venous thrombosis and pulmonary embolism in middle-aged men - A nested case-control study. *Thromb Haemost* 1997; 78: 1178-1182.
17. Forastiero RR, Martinuzzo ME, Cerrato GS, Kordich LC, Carreras LO. Relationship of anti β2-glycoprotein I and anti prothrombin antibodies to thrombosis and pregnancy loss in patients with antiphospholipid antibodies. *Thromb Haemost* 1997; 78: 1008-1014.
18. Galli M, Beretta G, Daldossi M, Bevers EM, Barbui T. Different anticoagulant and immunological properties of anti-prothrombin antibodies in patients with antiphospholipid antibodies. *Thromb Haemost* 1997; 77: 486-491.
19. Pengo V, Biasolo A, Brocco T, Tonetto S, Ruffatti A. Autoantibodies to phospholipid-binding plasma proteins in patients with thrombosis and phospholipid-reactive antibodies. *Thromb Haemost* 1996; 75: 721-724.
20. Arnout J, Wittevrongel C, Vanrusselt M, Hoylaerts M, Vermylen J. Beta-2-glycoprotein I dependent lupus anticoagulants form stable bivalent antibody beta-2-glycoprotein I complexes on phospholipid surfaces. *Thromb Haemost* 1998; 79: 79-86.
21. Lutters BCH, Meijers JCM, Derksen RHWM, Arnout J, De Groot PG. Dimers of β_2-glycoprotein I mimic the in vitro effects of β_2-glycoprotein I-anti-β_2-glycoprotein I antibody complexes. *J Biol Chem* 2001; 276: 3060-3067.
22. van Zanten HG, Saelman EUM, Schut Hese KM, Wu YP, Slootweg PJ, Nieuwenhuis HK, De Groot PG, Sixma JJ. Platelet adhesion to collagen type IV under flow conditions. *Blood* 1996; 88: 3862-3871.
23. Houdijk WPM, Sakariassen KS, Nievelstein PFEM, Sixma JJ. Role of factor VIII-von Willebrand Factor and fibronectin in the interaction of platelets in flowing blood with monomeric and fibrillar collagen types I and III. *J Clin Invest* 1985; 75: 531-540.
24. Reverter JC, Tàssies D. Mechanism of thrombosis in the antiphospholipid syndrome: Binding to platelets. In: Khamashta MA, ed. *The Hughes Syndrome*. London: Springer-Verlag; 2000:290-298.
25. Carreras LO, Forastiero R, Martinuzzo M. Interaction between antiphospholipid antibodies and eicosanoids. In: Khamashta MA, ed. *The Hughes Syndrome.* London: Springer-Verlag; 2000:337-347.
26. Meroni PL, Raschi E, Camera M, Testoni C, Nicoletti F, Tincani A, Khamashta MA, Balestrieri G, Tremoli A, Hess DC. Endothelial activation by aPL: A potential pathogenic mechanism for clinical manifestations of the syndrome. *J Autoimmun* 2000; 15: 237-240.
27. Zhao Y, Rumold R, Zhu M, Zhou D, Ahmed AE, Le DT, Hahn BH, Woods VL, Chen PP. An IgG antiphrothrombin antibody enhances prothrombin binding to damaged endothelial cells and shortens plasma coagulation times. *Arthritis Rheum* 1999; 42: 2132-2138.

28. Lutters BCH, Derksen RHWM, Tekelenburg WL, Lenting PJ, Arnout J, De Groot PG. An explanation for arterial thrombosis in the antiphospholipid syndrome: Dimers of β_2-glycoprotein I increase platelet deposition to collagen via interaction with phospholipids and a cellular receptor. 2002; Chapter 4: 63-82.
29. Simmelink MJ, Fernandez JA, Derksen RH, Griffin JH, De Groot PG. Low levels of activated protein C in patients with systemic lupus erythematosus do not relate to lupus anticoagulants but to low levels of factor II. *Br J Haematol* 2002; 117: 676-684.

Chapter 6

Anti-β_2-glycoprotein I antibodies do not affect platelet aggregation

Bianca C.H. Lutters[1,3], Ronald H.W.M. Derksen[2], Winnie L.Tekelenburg[1], Jef Arnout[4], Philip G. de Groot[1,3]

Departments of [1]Haematology and [2]Rheumatology and Clinical Immunology, University Medical Center, Utrecht and [3]Institute of Biomembranes, Utrecht University, The Netherlands, [4]Center for Molecular and Vascular Biology, University of Leuven, Leuven, Belgium.

Dear Sir,

In the antiphospholipid syndrome, antibodies against β_2-glycoprotein I (β_2GPI) are thought to play an important role. Anti-β_2GPI antibodies can prolong phospholipid-dependent coagulation assays, which is termed lupus anticoagulant (LAC) activity. Such LAC activity is associated with thrombophilia. We have previously shown that chimeric constructs of β_2GPI and the dimerization domain of coagulation factor XI, induce LAC activity in plasma and thus mimic effects of β_2GPI-anti-β_2GPI antibody complexes *in vitro* [1]. The same constructs induce increased platelet adhesion to collagen in an *in vitro* flow system, similar to most monoclonal and patient-derived LAC-positive anti-β_2GPI antibodies [2]. These findings indicate that anti-β_2GPI antibodies can influence the function of platelets, albeit that the exact mechanism by which they to so is not clear at present.

It has been reported that anti-β_2GPI antibodies stimulate platelet aggregation *in vitro* in the presence of low concentrations of an agonist such as ADP and collagen [3]. However, it was also described that LAC positive antibodies inhibited platelet aggregation induced by collagen [4]. Arvieux *et al.* [3] showed that the antibody-induced stimulation of aggregation does not occur when platelets are pre-incubated with monoclonal antibody IV.3, an antibody that blocks but does not activate the platelet FcγRII. This suggests that antibody-induced platelet aggregation includes an FcγRII-dependent mechanism. In contrast, Jankowski *et al.* [5] showed that platelet aggregation in the presence of low concentrations of ADP is stimulated by $F(ab)_2$ fragments of a monoclonal anti-β_2GPI antibody, indicating an FcγRII-independent mechanism of platelet activation. To further study this contradiction, we performed aggregation experiments in the presence of low concentrations of ADP with our dimeric β_2GPI constructs and with monoclonal anti-β_2GPI antibodies. Platelet-rich plasma (PRP) of healthy donors (with informed consent) was incubated for 5 min at 37°C with 200 µg/mL of dimeric construct or 100 µg/mL of monoclonal anti-β_2GPI antibody, followed by induction of aggregation with low concentrations of ADP (1 or 2 µM). In the presence of the dimeric β_2GPI constructs no effect on ADP-induced aggregation was observed (fig. 1A). However, we found that ADP-induced aggregation was increased in the presence of anti-β_2GPI antibodies (fig. 1B). This phenomenon disappeared when platelets were pre-incubated with 1 µg/mL of monoclonal antibody IV.3. These results are in accordance to the observations of Arvieux *et al.*[3] and suggest an FcγRII-dependent mechanism of platelet activation (fig. 1C). However, we previously found that in a perfusion model in which blood was flown over a collagen surface, that the increase in platelet adhesion in the presence of anti-β_2GPI antibodies was not dependent on the activation of the platelet FcγRII [2]. Therefore, we hypothesized that the stimulation of antibody-induced platelet aggregation could be caused by aggregates present in the anti-β_2GPI antibody solution. To test this hypothesis, we spinned down possible aggregates by centrifugation for 10 min at 10,000 g. Immediately thereafter, the effect of the antibodies on platelet aggregation was measured again. As shown in fig. 1D, centrifugation abolished the stimulatory effects of the antibody preparations on platelet activation. This suggests that indeed aggregates of IgG induced activation of the platelet FcγRII. The presence of aggregates of IgG molecules was further confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). We conclude that the effects of anti-β_2GPI antibodies on platelets in an aggregometer are not a consequence of dimerization of β_2GPI by these antibodies, but

are due to FcγRII activation by aggregates of IgG molecules present in the antibody preparations. Therefore, we feel that in all experiments with anti-β_2GPI antibodies and cells aggregated IgG should be removed. Jankowski *et al.* showed that anti-β_2GPI antibodies were able to stimulate platelet in an FcγRII-independent manner [5]. We think that these results can be explained by the use of a precisely chosen (donor-dependent) concentration of ADP.

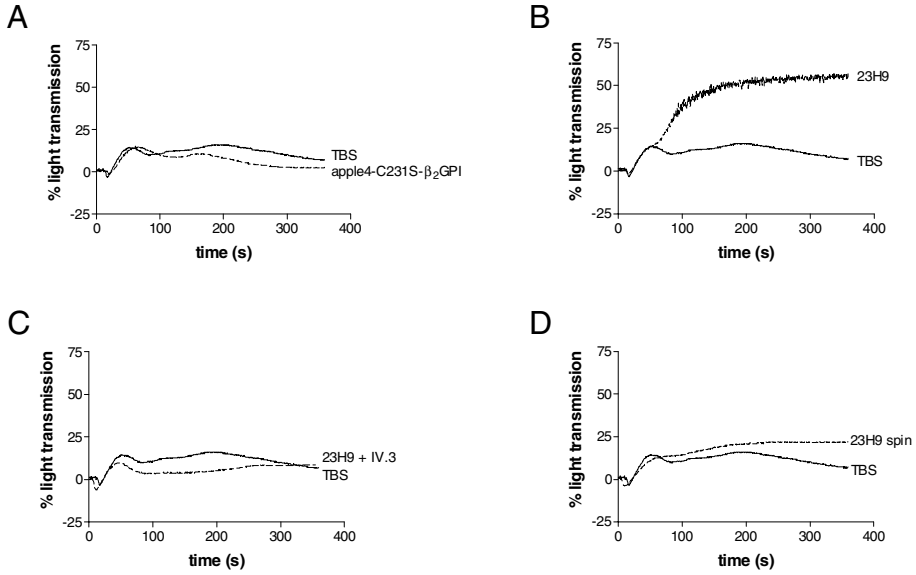

FIG.1. **Effects of dimers of β_2GPI and anti-β_2GPI antibodies on platelet aggregation.** Dimers of β_2GPI (*A*), monoclonal anti-β_2GPI antibody 23H9 (*B*), antibody 23H9 in combination with antibody IV.3 (*C*) or antibody 23H9 after centrifugation (*D*) were added to PRP. Afterwards aggregation was started with low concentrations of ADP and recorded.

References

1. Lutters BCH, Meijers JCM, Derksen RHWM, Arnout J, De Groot PG. Dimers of β_2-glycoprotein I mimic the in vitro effects of β_2-glycoprotein I-anti-β_2-glycoprotein I antibody complexes. *J Biol Chem* 2001; 276: 3060-3067.
2. Lutters BCH, Derksen RHWM, Tekelenburg WL, Lenting PJ, Arnout J, De Groot PG. An explanation for arterial thrombosis in the antiphospholipid syndrome: Dimers of β_2-glycoprotein I increase platelet deposition to collagen via interaction with phospholipids and a cellular receptor. 2002; Chapter 4: 63-82.
3. Arvieux J, Roussel B, Pouzol P, Colomb MG. Platelet activating properties of murine monoclonal antibodies to β_2-glycoprotein I. *Thromb Haemost* 1993; 70: 336-341.
4. Ostfeld I, Dadosh-Goffer N, Borokowski S, Talmon J, Mani A, Zor U, Lahav J. Lupus anticoagulant antibodies inhibit collagen-induced adhesion and aggregation of human platelets in vitro. *J Clin Immunol* 1992; 12: 415-423.
5. Jankowski M, Vreys I, Wittevrongel C, Boon D, Vermylen J, Hoylaerts MF, Arnout J. Thrombogenecity of beta2-glycoprotein I dependent antiphospholipid antibodies in a photochemically induced thrombosis model in the hamster. *Blood* 2002 [epub ahead of print].

Chapter 7

Binding of β_2-glycoprotein I and dimeric β_2-glycoprotein I to members of the low-density lipoprotein receptor superfamily: implications for the antiphospholipid syndrome

Bianca C.H. Lutters[1,3], Monique van Scherpenzeel[1], Ronald H.W.M. Derksen[2], Peter J. Lenting[1,3], Philip G. de Groot[1,3]

Departments of [1]Haematology and [2]Rheumatology and Clinical Immunology, University Medical Center, Utrecht and [3]Institute of Biomembranes, Utrecht University, The Netherlands.

Abstract

At present, the mechanisms by which anti-β_2GPI antibodies can cause thrombosis in patients with the antiphospholipid syndrome is not clear. It seems conceivable that this mechanism involves a receptor-dependent process. Megalin, a member of the low-density lipoprotein (LDL) receptor family, has been described as a renal clearance receptor for β_2GPI [1]. In the present study, we compared binding of β_2GPI to megalin and its structural homologue low-density lipoprotein receptor-related protein (LRP). Both monomeric and dimeric β_2GPI bind to megalin, although binding of dimeric β_2GPI proved to be more efficient. In contrast, dimeric β_2GPI, but not monomeric β_2GPI interacts with LRP. This interaction could be described by a model involving two binding sites. Replacement of Trp at position 316 by Ser in domain V of β_2GPI resulted in reduced binding to LRP, which shows involvement of domain V of β_2GPI. Furthermore, an anti-β_2GPI antibody directed against a region located at the interface of domain I and II of β_2GPI inhibited association of dimeric β_2GPI to LRP, suggesting that this region of β_2GPI also has a role in the interaction between dimeric β_2GPI and LRP. The involvement of both domain V and the interface of domain I and II of β_2GPI was supported by the observation that heparin, a molecule that can interact with both regions, inhibited the association of dimeric β_2GPI to LRP completely.

Introduction

In the antiphospholipid syndrome, the presence of autoantibodies against β_2GPI is thought to be the cause of the increased risk for thrombo-embolic complications. The presence of these antibodies can result in a prolongation of clotting time in phospholipid-dependent coagulation assays, an activity termed lupus anticoagulant (LAC). In order to study the molecular mechanism of β_2GPI-anti-β_2GPI antibody complexes, we constructed chimeric fusion proteins between the dimerization domain of coagulation factor XI (apple4) and β_2GPI and used these as a model for β_2GPI-anti-β_2GPI antibody complexes. We previously showed that these constructs have LAC activity [2] and that their presence can increase collagen-induced platelet adhesion and aggregate formation [3]. This increase in platelet adhesion was also observed in the presence of LAC-positive anti-β_2GPI antibodies [3]. As binding to phospholipids alone could not explain the activation of platelets, we proposed that the mechanism by which β_2GPI-anti-β_2GPI antibody complexes can exert their pathogenic effects involves a receptor-dependent process. In agreement with this model, we showed that the increase in platelet adhesion to collagen in the presence of dimeric β_2GPI or anti-β_2GPI antibodies was inhibited in the presence of receptor-associated protein (RAP). RAP blocks binding of ligands to members of the LDL-receptor family.

β_2-glycoprotein I (β_2GPI) is a member of the complement control protein (CCP) or short consensus repeat (SCR) superfamily. It consists of five domains, of which the N-terminal four domains comprise ~60 amino acids and contain 2 disulfide bonds. The C-terminal domain is dissimilar in that it contains an extra C-terminal loop and 3 disulfide bonds. This domain harbours the phospholipid binding site [4-8]. At present, two cellular receptors for β_2GPI have been identified: megalin and annexin II. Annexin II is present on endothelial cells, where it functions as a receptor for tissue-type plasminogen activator (tPA) and plasminogen [9]. The physiological relevance of the interaction between β_2GPI and annexin II is remains yet to be determined. Megalin, a member of the low-density lipoprotein (LDL) receptor superfamily, was identified as the renal clearance receptor for β_2GPI [1].

Megalin has structural homology with the low-density lipoprotein receptor-related protein (LRP) [10-13], also a member of the LDL-receptor family. Furthermore, *in vitro* megalin binds many ligands that are also known to bind to LRP, including lipoproteins, and urokinase [11]. Therefore, we determined the ability of β_2GPI and the dimers of β_2GPI to bind to megalin and LRP. We showed that β_2GPI and dimers of β_2GPI can bind to megalin. In contrast, dimers of β_2GPI, but not monomeric β_2GPI, associate to LRP. Finally, experiments using mutant dimeric β_2GPI and inhibitors of the interaction between dimeric β_2GPI and LRP suggest that this particular interaction involves domain V and the interface of domain I and II of β_2GPI.

Materials and methods

Construction and expression of recombinant proteins

The chimeric dimer apple4-C321S-β_2GPI was constructed as described previously [2]. As a control, the dimer apple4-C321S-β_2GPI-W316, in which β_2GPI-W316S is not able to bind to phospholipids, was made. To exclude the possibility that apple4-β_2GPI binds via its apple domain to LRP and megalin, dimer apple4 was constructed. The sequence of dimer apple4 was amplified from the vector apple4-tissue-type plasminogen activator (tPA)-S478A [14] with the primers apple4-BglII GCC AGA TCT TTC TGC CAT TCT TCA and apple4-XbaI GGT CTA GAC TCG AGT CCC TCC TTT GAT GCG TG. The PCR product was subcloned into the vector pCR®2.1-TOPO® (Invitrogen, Breda, The Netherlands), and cloned into the vector apple4-tPA-S478A with BglII and XbaI (underlined in apple4-BglII and apple4-XbaI, respectively). Sequence analysis was performed to confirm correct amplification of apple4. The proteins were expressed by stably transfected baby hamster kidney cells [2]. Expression of constructs was performed in conditioned serum-free medium (DMEM F12 supplemented with 1% UltroserG; Life Technologies, Inc., Paisley, U.K.).

Purification of proteins and antibodies

Plasma-derived β_2GPI was purified from human plasma as described by Horbach et al. [15]. Chimeric fusion proteins were purified using a monoclonal antibody to β_2GPI (21B2) bound to CNBr-activated Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden)[8]. Bound fusion proteins were eluted with 0.1 M glycine (pH 2.7). The purified proteins were immediately neutralized with 1 M Tris, pH 9. Àpple4-C321S- β_2GPI and apple4-C321S-β_2GPI-W316S containing fractions were dialyzed against 50 mM Na_2HPO_4, pH 7.0, containing 50 mM NaCl and were subjected to further purification on a mono S column using FPLC (Amersham Pharmacia Biotech, Uppsala, Sweden). Fusion proteins were eluted with a linear salt gradient from 50 mM NaCl to 1 M NaCl. All chimera were dialyzed against tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4). Apple4 was purified using monoclonal antibody XI-1, which recognizes the apple4 domain [16], coupled to CNBr-activated Sepharose. Bound proteins were eluted with 0.1 M glycine (pH 2.7). The purified proteins were immediately neutralized with 1 M Tris, pH 9. Monoclonal anti-β_2GPI antibody 4F3, monoclonal antibody XI-1 and IgG from pooled normal plasma were purified using a protein G column. Bound antibodies were eluted with 0.1 M glycine, pH 2.7, neutralized with 1 M Tris, pH 9, and dialyzed against TBS. Monoclonal anti-β_2GPI antibodies 22A11, 23H9 and 19H9 were described previously [2,17] and monoclonal anti-β_2GPI antibodies 1F12 and 2B2 were kindly provided by Dr. A. Tincani (Clinical Immunology Unit, Spedali Civili, Brescia, Italy). LRP and megalin were kindly provided by Dr. S.K. Moestrup (University of Aarhus, Aarhus, Denmark). Protein concentration was determined using a bichinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) according to the instructions of the manufacturer, and with bovine serum albumin (BSA) as a standard. Purified proteins were analyzed by 4-15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Dimer apple4 migrated under reducing conditions at 10 kDa and under non-reducing conditions at 20 kDa, as was expected.

Surface plasmon resonance analysis

Binding studies were performed using a Biacore2000 biosensor system (Biacore AB, Uppsala, Sweden) and surface plasmon resonance (SPR) analysis was performed as described [18,19]. LRP or megalin was immobilized on a CM5 sensor chip using the amine-coupling kit according to the instructions of the manufacturer (Biacore AB, Uppsala, Sweden). One channel was activated and blocked in the absence of protein and afterwards its signal (<5% of binding to the coated channels) was used to correct the signal from the coated channels for aspecific binding. TBS with 0.005% (v/v) Tween 20 was used as flow buffer. Proteins were diluted in flow buffer with 1 μM $CaCl_2$. SPR analysis was performed at 25°C and at a flow rate of 10 μL/min. To determine the association and dissociation constants for the interaction of dimers of β_2GPI with immobilized LRP a flow rate of 30 μL/min was used. Regeneration of the surfaces of the chips were performed with 100 mM H_3PO_4 for immobilized LRP and with 0.1 M sodium-citrate containing 1mM of EDTA and 1 M of NaCl, pH 5.0 for immobilized megalin.

Analysis of SPR data

For analysis of the association and dissociation curves of the sensograms, BiaEvaluation software (Biacore AB, Uppsala, Sweden) was used. Interaction constants were determined by performing non-linear global fitting of data corrected for bulk refractive index changes. Data were fitted to various models available within the software. For binding of dimer β_2GPI to LRP, a model describing the interaction between dimer β_2GPI and two independent binding sites (heterologous ligand, parallel reactions) was found to provide the best fit for the experimental data. Accuracy of the fits was judged from residual plots and statistical parameters from previously described equations [20].

Mapping of the epitope recognized by monoclonal anti-β_2GPI antibodies on LRP

Recombinant fragments of β_2GPI (kindly provided by Dr. G.M. Iverson, La Jolla Pharmaceutical Company, San Diego, USA)[21] were coated overnight at 4°C at a concentration of 10 μg/mL in TBS on high binding ELISA plates (Costar, stripwell plate, 9102 cat. no., Corning Incorporated, Corning, NY, USA). Plates were washed three times with TBS/0.1% Tween 20 and blocked with 150 μL/well TBS/ 0.1% gelatin/ 0.5 mg/mL purified horse IgG/ 0.1% Tween 20 for 1 h at 37°C. After three times washing, plates were incubated with 10 μg/mL of the monoclonal antibody (50 μL/well) for 2 h at 37°C. Plates were washed and subsequently incubated with a peroxidase-labeled rabbit-anti-mouse antibody (RAMPO; Dako, Glostrup, Denmark), diluted 1:1000 (50 μL/well), for 1 h at 37°C. Development of the ELISA was performed using o-phenylene-diamine (OPD).

Results

Binding of dimeric β_2GPI to megalin

It previously has been shown that megalin acts as a receptor for β_2GPI, and that receptor binding is enhanced by the association of β_2GPI to a negatively charged phospholipid surface [1]. We therefore addressed the question whether enhanced receptor binding could be a result of dimerization of β_2GPI. To this end, monomeric and dimeric β_2GPI were compared for binding to megalin by SPR analysis. At a concentration of 3.8 μM β_2GPI, reversible binding to megalin (25 fmol/mm^2) was observed (fig. 1, line III). However, at lower concentrations of β_2GPI (0.1 μM), β_2GPI association to megalin could not be detected (fig. 1, IV), suggesting that complex formation involves a low affinity interaction. At a similar concentration of 0.1 μM, efficient binding of a dimeric variant of β_2GPI (i.e. apple4-C321S-β_2GPI fusion protein [2]) to megalin was detected (fig. 1, line I). It also should be noted that the apple4 domain of factor XI that mediates dimerization of β_2GPI in the fusion protein displayed association to megalin, although to a minor extent (fig. 1, line II). Nevertheless, our data indicate that dimerization of β_2GPI may increase the association efficiency of the ligand to the receptor.

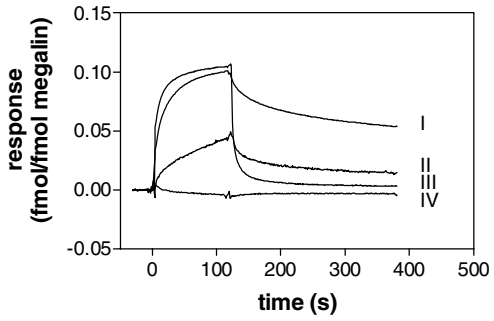

FIG. 1. **Binding of apple-β_2GPI fusion proteins to immobilized megalin.** Megalin was immobilized on CM5 sensor chips at a density of 25 fmol/mm^2. The protein was incubated for 2 min at a flow rate of 10 μL/min with the various proteins at 25°C. Ligand solution was replaced with buffer (TBS/0.005% Tween 20) to initiate dissociation. I, apple4-C321S-β_2GPI (0.1 μM); II, apple4 (0.1 μM); III, β_2GPI (3.8 μM); IV, β_2GPI (0.1 μM).

Binding of dimeric β_2GPI to LRP

Although megalin and LRP share a number of ligands, the β_2GPI monomer seems to interact with megalin selectively [1]. Indeed, by using concentrations as high as 3.8 μM, no association of β_2GPI to LRP (8 fmol/mm2) could be detected (fig. 2, line IV). In contrast, dimeric β_2GPI (0.1 μM) displayed reversible binding to immobilized LRP, whereas no binding of dimeric apple4 (0.1μM) was observed (fig. 2, line I and III, respectively). We studied the binding of dimeric β_2GPI to LRP in more detail by assessing the apparent association and dissociation rate constants, which are summarized in table 1. Data analysis revealed that a model describing the interaction of dimers of β_2GPI with two classes of binding sites (heterologous ligand, parallel interactions) provided the best fit for the experimental data [20]. By using this model, a high and low affinity interaction with apparent affinity constants of approximately 3 and 54 nM could be inferred from the kinetic data.

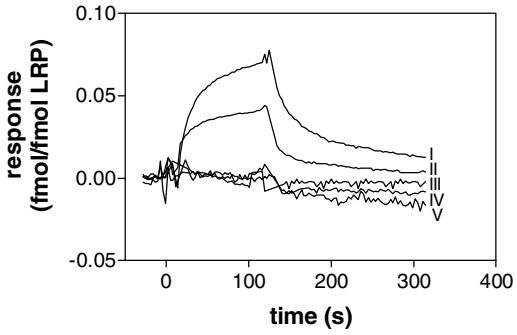

FIG. 2. **Interaction of apple-β_2GPI fusion proteins with immobilized LRP.** Proteins were allowed to interact with LRP (8 fmol/mm^2) for 2 min at a flow rate of 30 µL/min. Afterwards, protein solution was replaced by buffer solution. I, apple4-C321S-β_2GPI (100 nM); II, apple4-C321S-β_2GPI-W316S (0.1 µM); III, apple4 (0.1 µM); IV, β_2GPI (3.8 µM); V, β_2GPI (0.1 µM).

TABLE 1. **Kinetic parameters for the binding of dimers of β_2GPI to LRP.** To calculate association rate constants ($k_{on(app)}$) and dissociation rate constants ($k_{off(app)}$), the data obtained for the interaction of various concentrations of dimeric β_2GPI with LRP were analyzed using a two-site binding model. 1 and 2 represent the two classes of binding sites. Apparent affinity constants ($K_{d(app)}$) were calculated from $k_{off(app)}$/$k_{on(app)}$. Data are based on three measurements using five different concentrations of dimeric β_2GPI for each measurement. Data represent mean (± SD).

	apple4-C321S-β_2GPI	apple4-C321S-β_2GPI-W316S
$k_{on(app)}$ (M-1s-1)		
1.	2.7 (± 0.4) x 10^6	6.0 (± 1.1) x 10^6
2.	2.8 (± 0.2) x 10^6	2.7 (± 0.3) x 10^5
$k_{off(app)}$ (M-1s-1)		
1.	0.14 (± 0.01)	0.44 (± 0.07)
2.	9.5 (± 1.2) x 10^{-3}	1.2 (± 0.8) x 10^{-2}
$K_{d(app)}$ (M-1s-1)		
1.	54 (± 8.0)	87 (± 49)
2.	3.2 (± 2.1)	51 (± 26)

Effect of a mutation in the phospholipid binding site of β_2GPI on binding to LRP

To investigate the possibility that binding of dimeric β_2GPI to LRP has similar characteristics as binding to negatively charged phospholipids, SPR analysis with a dimer of β_2GPI that is unable to bind to phospholipids (apple4-C321S-β_2GPI-W316S) was performed. As shown in fig. 2 (line II), this dimer associated to immobilized LRP, although the maximal response was lower than the response for the association of apple4-C321S-β_2GPI to LRP. As shown in table 1, the interaction between apple4-C321S-β_2GPI-W316S and LRP (apparent affinity constants 51 ± 26 nM and 87 ± 49 nM) only showed low affinity binding. These constants differ from the constants for the interaction between dimer β_2GPI and LRP. The most dominant effect was observed on the association rate constant referred to as 2, which was found to be 10-fold lower for apple4-C321S-β_2GPI-W316S (2.7 (± 0.3) x 10^5 $M^{-1}s^{-1}$) than for apple4-C321S-β_2GPI (2.8 (± 0.2) x 10^6 $M^{-1}s^{-1}$). Our data with the mutant dimer indicate that

replacement of Trp by Ser at position 316 in β_2GPI reduces the affinity of dimeric β_2GPI for LRP, suggesting that domain V may contribute to interaction with LRP.

Binding of dimer β_2GPI-anti-β_2GPI antibody complexes to LRP

To further characterize the interaction between dimeric β_2GPI and LRP, we used an array of monoclonal anti-β_2GPI antibodies. To do so, apple4-C321S-β_2GPI (0.1 μM) was incubated with various monoclonal anti-β_2GPI antibodies (0.1 μM) for 30 min at 37°C. Afterwards, the interaction of the dimeric β_2GPI-anti-β_2GPI antibody complexes with immobilized LRP was assessed. In the presence of antibody 4F3, binding of apple4-C321S-β_2GPI to immobilized LRP was inhibited (50%) (fig. 3). Anti-β_2GPI antibody 1F12 also inhibited the binding of dimeric β_2GPI to LRP, although the inhibition was less strong than observed with antibody 4F3 (20% inhibition). Antibodies 23H9, 22A11, 2B2 and 19H9 did not inhibit the binding of dimeric β_2GPI to LRP.

As we are interested in the epitopes on β_2GPI involved in the binding to LRP, the epitopes on β_2GPI that are recognized by the monoclonal anti-β_2GPI antibodies were determined. Therefore, recombinant fragments of β_2GPI were coated and binding of the antibodies was determined using an ELISA-setup. Domain I, domains I-II, domains I-II-III, domains I-II-III-IV, domain V, domains IV-V, domains III-IV-V, domains II-III-IV-V and wild-type β_2GPI were used [21]. As shown in table 2, the combined presence of domains I and II is needed for the binding of monoclonal anti-β_2GPI antibody 4F3 to β_2GPI, suggesting that this antibody is directed against an epitope located at the interface of domain I and II. Both 23H9 and 22A11 were found to be directed against an epitope on domain II of β_2GPI, and antibody 2B2 is directed against a region located on domain III. The epitopes recognized by antibodies 19H9 and 1F12 were shown to be located on domain IV and at the interface of domains IV and V of β_2GPI, respectively. Thus, these results suggest that besides domain V, also a region at the interface of domains I and II has a role in the binding of dimeric β_2GPI to LRP.

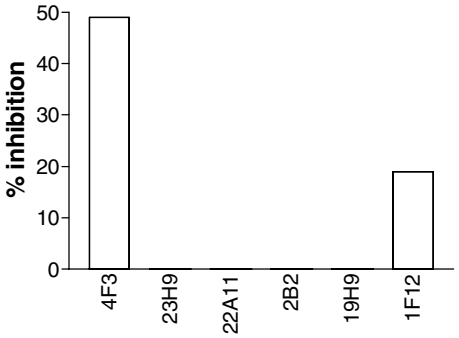

FIG. 3. **Effect of anti-β_2GPI antibodies on the interaction between apple4-C321S-β_2GPI and LRP.** Apple4-C321S-β_2GPI (0.1 μM) was preincubated for 30 min at 37°C with 0.1 μM of anti-β_2GPI antibodies. Afterwards, the effect of preincubation with these antibodies on the binding of dimeric β_2GPI to immobilized LRP (4 fmol/mm^2) was investigated using SPR analysis. Binding is expressed as percentage of inhibition dimeric β_2GPI alone to LRP.

TABLE 2. **Mapping of the epitopes recognized by anti-β_2GPI antibodies on β_2GPI.** An ELISA-setup, in which domain-deleted mutants of β_2GPI were coated, was used to determine the binding of anti-β_2GPI antibodies to domains of β_2GPI.

	4F3	23H9	22A11	2B2	19H9	1F12
domain I	-	-	-	-	-	-
domains I-II	+	+	+	-	-	-
domains I-II-III	+	+	+	+	-	-
domains I-II-III-IV	+	+	+	+	+	-
domain V	-	-	-	-	-	-
domains IV-V	-	-	-	-	+	+
domains III-IV-V	-	-	-	+	+	+
domains II-III-IV-V	-	+	+	+	+	+
wild-type	+	+	+	+	+	+

Effects of heparin on the interaction between dimeric β_2GPI and LRP

β_2GPI contains two regions enriched in positively charged amino acid residues. These domains appear to be located in domain V and in domain I (located near the interface of domain I and II) and are involved in the binding of heparin [22,23]. As we showed that both regions might contribute to the interaction between dimeric β_2GPI and LRP, we investigated the ability of heparin to inhibit this interaction. Therefore, apple4-C321S-β_2GPI (0.1 μM) was incubated with various concentrations of heparin for 30 min at 37°C. Subsequently, binding of the complex between dimeric β_2GPI and heparin to LRP was measured using SPR analysis. As shown in fig. 4, heparin inhibited the binding of apple4-C321S-β_2GPI to LRP (>95%). Half maximal inhibition of the association of dimeric β_2GPI to LRP was observed at a concentration of 13 μg/mL of heparin.

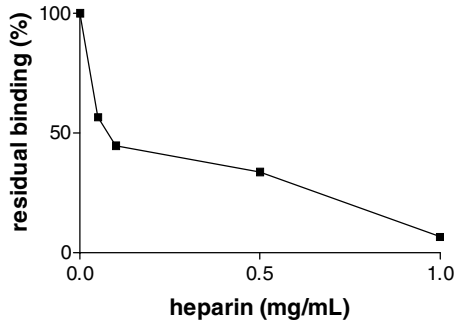

FIG. 4 **Effect of heparin on the interaction between dimeric β_2GPI and immobilized LRP.** Apple4-C321S-β_2GPI (0.1 μM) was preincubated for 30 min at 37°C with various concentrations of heparin. This was followed by determination of the ability of apple4-C321S-β_2GPI to bind to LRP (4 fmol/mm^2) in the presence or absence of heparin by SPR analysis.

Discussion

The mechanism by which anti-β_2GPI antibodies cause thrombosis is not known at present. We have previously proposed a model for arterial thrombosis in which the β_2GPI-anti-β_2GPI antibody complexes bind to negatively charged phospholipids exposed at the surface of activated platelets, followed by the interaction of the complex with a receptor specific for β_2GPI. As our results suggested that a receptor of the LDL receptor family was involved [3], we focussed on the interaction of dimeric β_2GPI with members of the LDL receptor family. We now showed that dimeric β_2GPI binds to LRP and megalin and that this interaction is reversible (fig. 1 and 2). At a concentration of 3.8 μM, which is similar to the concentration in plasma, binding of monomeric β_2GPI to megalin was observed. In contrast, β_2GPI did not interact with LRP at a similar concentration. An interesting observation was that the apple4 dimer interacted with megalin, but not with LRP. Since apple4 is the dimerization domain of factor XI, this observation suggests that factor XI might also associate to megalin. SPR analysis indeed showed that factor XI interacts with megalin (results not shown), although the physiological relevance of this interaction is not clear yet. As it has been shown that both factor XI and activated factor XI failed to associate to LRP [19], this is again a ligand that only interacts with megalin and not with LRP.

The interaction between dimeric β_2GPI and immobilized LRP was studied in more detail by using a mutant dimeric β_2GPI that cannot bind to negatively charged phospholipids, a panel of monoclonal anti-β_2GPI antibodies and heparin. Hereby, it was shown that a region at the interface of domain I and II and a region in domain V of β_2GPI participate in the association of dimeric β_2GPI to LRP. Besides these two sites on β_2GPI, LRP also possesses two possible interaction sites that also can be involved in this interaction: ligand binding clusters II and IV [24]. Furthermore, it should be noted that current software has limitations in that it is not possible to use more complicated models for kinetic analysis of the data. Therefore, it is impossible to discriminate between the different interaction sites, implicating that all values have to be interpreted as apparent values.

In an *in vitro* flow model for arterial thrombosis, we have shown that two LAC positive monoclonal anti-β_2GPI antibodies (4F3 and 2B2) had no effect on platelet adhesion to collagen, while the other LAC positive antibodies increased platelet adhesion to collagen. We hypothesized that 4F3 and 2B2 interfered with the binding of the β_2GPI-anti-β_2GPI antibody to a cellular receptor. Our hypothesis was supported by the finding that antibody 4F3 inhibited the increase in platelet adhesion to collagen induced by dimeric β_2GPI [3]. Here, we showed that 4F3 indeed inhibits the interaction between dimeric β_2GPI and LRP. In contrast, antibody 2B2 did not inhibit this association (fig. 3). An explanation for this observation might be that it is not LRP that is located at the platelet membrane, but an other member of the LDL-receptor family, a splice variant of LRP8 [25]. Antibodies 23H9, 22A11 and 19H9 also did not interfere with the interaction between dimeric β_2GPI and LRP. As LRP is expressed on the membrane of a large number of cell types, it may have a role in the development of thrombosis in the antiphospholipid syndrome. Two cell types that have been described to involved in the pathogenesis of the antiphospholipid syndrome have LRP located at their cell surface: monocytes and cells of the syncytiotrophoblast, a part of the placenta [12]. Therefore, it would be interesting to determine the role of LRP in the activation of these cells by β_2GPI-anti-β_2GPI

antibody complexes. The data presented here fit nicely with the model we proposed for the development of an arterial thrombus, although the mechanism by which these cells are thrombotic is different to that by which platelets exert their thrombotic effects. When monocytes and cells of the placenta interact with β_2GPI-anti-β_2GPI antibody complexes, they become activated and express thrombotic and anti-thrombotic molecules on their surface. By the exposure of these molecules on their surfaces, the development of a thrombus may be stimulated.

References

1. Moestrup SK, Schousboe I, Jacobsen C, Leheste JR, Christensen EI, Willnow TE. β_2-glycoprotein I (apolipoprotein H) and β_2-glycoprotein I-phospholipid complex harbor a recognition site for the endocytic receptor megalin. *J Clin Invest* 1998; 102: 902-909.
2. Lutters BCH, Meijers JCM, Derksen RHWM, Arnout J, De Groot PG. Dimers of β_2-glycoprotein I mimic the in vitro effects of β_2-glycoprotein I-anti-β_2-glycoprotein I antibody complexes. *J Biol Chem 2001*; 276: 3060-3067.
3. Lutters BCH, Derksen RHWM, Tekelenburg WL, Lenting PJ, Arnout J, De Groot PG. An explanation for arterial thrombosis in the antiphospholipid syndrome: Dimers of β_2-glycoprotein I increase platelet deposition to collagen via interaction with phospholipids and a cellular receptor. 2002; Chapter 4: 63-82.
4. Hunt JE, Simpson RJ, Krilis SA. Identification of a region of β_2-glycoprotein I critical for lipid binding and anti-cardiolipin antibody cofactor activity. *Proc Natl Acad Sci USA* 1993; 90: 2141-2145.
5. Hunt J, Krilis S. The fifth domain of β_2-glycoprotein I contains a phospholipid binding site (Cys281-Cys288) and a region recognized by anticardiolipin antibodies. J Immunol 1994; 152: 653-659.
6. Sheng YH, Sali A, Herzog H, Lahnstein J, Krilis SA. Site-directed mutagenesis of recombinant human β_2-glycoprotein I identifies a cluster of lysine residues that are critical for phospholipid binding and anti-cardiolipin antibody activity. *J Immunol* 1996; 157: 3744-3751.
7. Mehdi H, Naqvi A, Kamboh MI. A hydrophobic sequence at position 313-316 (Leu-Ala-Phe-Trp) in the fifth domain of apolipoprotein H (β_2-glycoprotein I) is crucial for cardiolipin binding. *Eur J Biochem* 2000; 267: 1770-1776.
8. Horbach DA, Van Oort E, Tempelman MJ, Derksen RHWM, De Groot PhG. The prevalence of a non-phospholipid-binding form of β_2-glycoprotein I in human plasma. Consequences for the development of anti-β_2-glycoprotein I antibodies. *Thromb Haemost* 1998; 80: 791-797.
9. Ma K, Simantov R, Zhang J-C, Silverstein R, Hajjar KA, McCrae KR. High affinity binding of β_2-glycoprotein I to human endothelial cells is mediated by annexin II. *J Biol Chem* 2000; 275: 15541-15548.
10. Strickland DK, Gonias SL, Argraves WS. Diverse roles for the LDL receptor family. *Trends Endocrinol Metab* 2002; 13: 66-74.
11. Willnow TE. The low density lipoprotein receptor gene family: multiple roles in lipid metabolism. *J Mol Med* 1999; 77: 306-315.
12. Neels JG, Horn IR, van den Berg BMM, Pannekoek H, Van Zonneveld A-J. Ligand-receptor interactions of the low density lipoprotein receptor-related protein, a multi-ligand endocytic receptor. *Fibrinol Proteol* 1998; 12: 219-240.
13. Willnow TE, Nykjær A, Herz J. Lipoprotein receptors: new roles for ancient proteins. *Nat Cell Biol* 1999; 1: E157-E162.
14. Meijers JC, Mulvihill ER, Davie EW, Chung DW. Apple four in human blood coagulation factor XI mediates dimer formation. *Biochemistry* 1992; 31: 4684.
15. Horbach DA, Van Oort E, Donders RCJM, Derksen RHWM, De Groot PG. Lupus

anticoagulant is the strongest risk factor for both venous and arterial thrombosis in patients with systemic lupus erythematosus - Comparison between different assays for the detection of antiphospholipid antibodies. *Thromb Haemost* 1996; 76: 916-924.
16. Meijers JCM. Regulation of the blood coagulation mechanism by plasma proteinase inhibitors. *PhD Thesis, Utrecht University, The Netherlands* 1988: 93-108.
17. Arnout J, Wittevrongel C, Vanrusselt M, Hoylaerts M, Vermylen J. Beta-2-glycoprotein I dependent lupus anticoagulants form stable bivalent antibody beta-2-glycoprotein I complexes on phospholipid surfaces. *Thromb Haemost* 1998; 79: 79-86.
18. Lenting PJ, Neels JG, van den Berg BMM, Clijsters PPFM, Meijerman DWE, Pannekoek H, Van Mourik JA, Mertens K, Van Zonneveld A-J. The light chain of factor VIII comprises a binding site for low density lipoprotein receptor-related protein. *J Biol Chem* 1999; 274: 23734-23739.
19. Neels JG, van den Berg BM, Mertens K, ter Maat H, Pannekoek H, Van Zonneveld A-J, Lenting PJ. Activation of factor IX zymogen results in exposure of a binding site for low-density lipoprotein receptor-related protein. *Blood* 2000; 96: 3459-3465.
20. Horn IR, van den Berg BM, van der Meijden PZ, Pannekoek H, Van Zonneveld A-J. Molecular analysis of ligand binding to the second cluster of complement-type repeats of the low density lipoprotein receptor-related protein. Evidence for an allosteric component in receptor-associated protein-mediated inhibition of ligand binding. *J Biol Chem* 1997; 272: 13608-13613.
21. Iverson GM, Victoria EJ, Marquis DM. Anti-β2 glycoprotein I (β2GPI) autoantibodies recognize an epitope on the first domain of β2GPI. *Proc Natl Acad Sci USA* 1998; 95: 15542-15546.
22. Guerin J, Sheng Y, Reddel S, Iverson GM, Chapman MG, Krilis SA. Heparin inhibits the binding of β_2-glycoprotein I to phospholipids and promotes the plasmin-mediated inactivation of this blood protein. Elucidation of the consequences of the two biological events in patients with the anti-phsopholipid syndrome. *J Biol Chem* 2002; 277: 2644-2649.
23. Hagihara Y, Goto Y, Kato H, Yoshimura T. Role of the N- and C-terminal domains of bovine β_2-glycoprotein I in its interaction with cardiolipin. *J Biochem (Tokyo)* 1995; 118: 129-136.
24. Neels JG, van den Berg BM, Lookene A, Olivecrona G, Pannekoek H, Van Zonneveld A-J. The second and fourth cluster of class A cysteine-rich repeats of the low-density lipoprotein receptor-related protein share ligand-binding properties. *J Biol Chem* 1999; 274: 31305-31311.
25. Riddell DR, Vinogradov DV, Stannard AK, Chadwick N, Owen JS. Identification and characterization of LRP8 (apoER2) in human blood platelets. *J Lip Res* 1999; 40: 1925-1930.

Chapter 8 | General discussion

The association between the persistent presence of antiphopsholipid 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 β_2-glycoprotein I (β_2GPI) 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-β_2GPI 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 β_2GPI, 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-β_2GPI 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-β_2GPI antibodies can cause thrombosis has never been described.

In this thesis, using fusion proteins of β_2GPI and the dimerization domain of coagulation factor XI as a model for β_2GPI-anti-β_2GPI antibody complexes, an *in vitro* thrombosis model for the antiphospholipid syndrome was developed to investigate a mechanism by which antiphospholipid antibodies can cause thrombosis.

β_2GPI-anti-β_2GPI complexes

Two mechanisms have been described by which anti-β_2GPI antibodies can form a complex with β_2GPI and subsequently exert their *in vitro* effects. First, one anti-β_2GPI antibody binds two molecules β_2GPI, thereby forming a bivalent complex [6-8] (fig. 1A), and second one anti-β_2GPI antibody binds one β_2GPI molecule, which results in a conformational change in β_2GPI [9-15] (fig. 1B). Both these mechanisms result in an enormously increased affinity for negatively charged phospholipids compared to β_2GPI alone. Using our chimeric constructs of β_2GPI and the dimerization domain of coagulation factor XI, we have shown that the formation of a bivalent complex between β_2GPI and anti-β_2GPI antibodies is enough to mimic the effects of β_2GPI-anti-β_2GPI 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-β_2GPI antibody complexed to β_2GPI 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 β_2GPI in solution has a slightly different

conformation compared to β_2GPI bound to a membrane [16]. 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 β_2GPI induces a conformational change in the domain V, which harbours the site that is essential for binding to phospholipids of β_2GPI. However, this is unlikely, because fusion of β_2GPI with a comparable domain, apple2 of factor XI, did not induce such a conformational change.

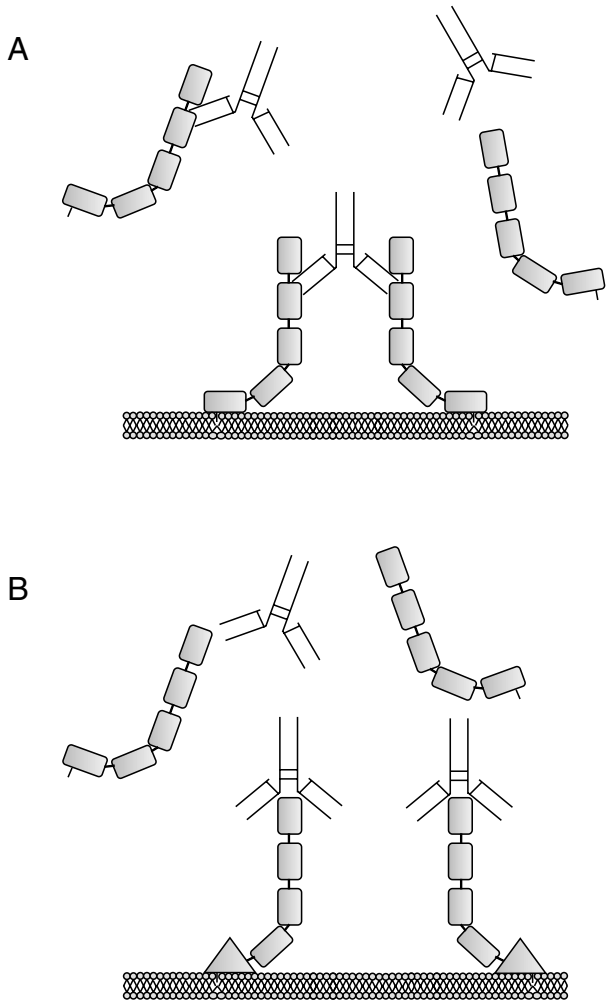

FIG.1. **Binding of β_2GPI-anti-β_2GPI antibody complexes to phospholipid membranes.** This can occur by the formation of a bivalent complex between β_2GPI and an anti-β_2GPI antibody (*A*) or by the induction of a conformational change in β_2GPI after binding to an anti-β_2GPI antibody (*B*). Both result in a highly increased affinity for phospholipids compared to β_2GPI alone.

A mechanism for the development of thrombosis

It has been shown that antiphospholipid antibodies, in particular anti-β_2GPI 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 β_2GPI-anti-β_2GPI 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-β_2GPI antibody and the complete anti-β_2GPI 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 β_2GPI and anti-β_2GPI 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 β_2GPI or β_2GPI-anti-β_2GPI 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 β_2GPI-anti-β_2GPI antibody complexes can cause (arterial) thrombosis. As shown in fig. 2, β_2GPI-anti-β_2GPI 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 β_2GPI-anti-β_2GPI 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 β_2GPI 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 β_2GPI-anti-β_2GPI antibody complexes exert their pathogenic effects. Furthermore, we showed that binding of dimeric β_2GPI to LRP is inhibited by anti-β_2GPI 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 β_2GPI 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 β_2GPI. For example, megalin can bind β_2GPI alone at a concentration of 0.2 μM, which is 20 times lower than the plasma concentration of β_2GPI. It has been suggested that megalin has a role in the clearance of phosphatidylserine-containing particles [28]. Recently, it was reported that β_2GPI 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 β_2GPI 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 β_2GPI.

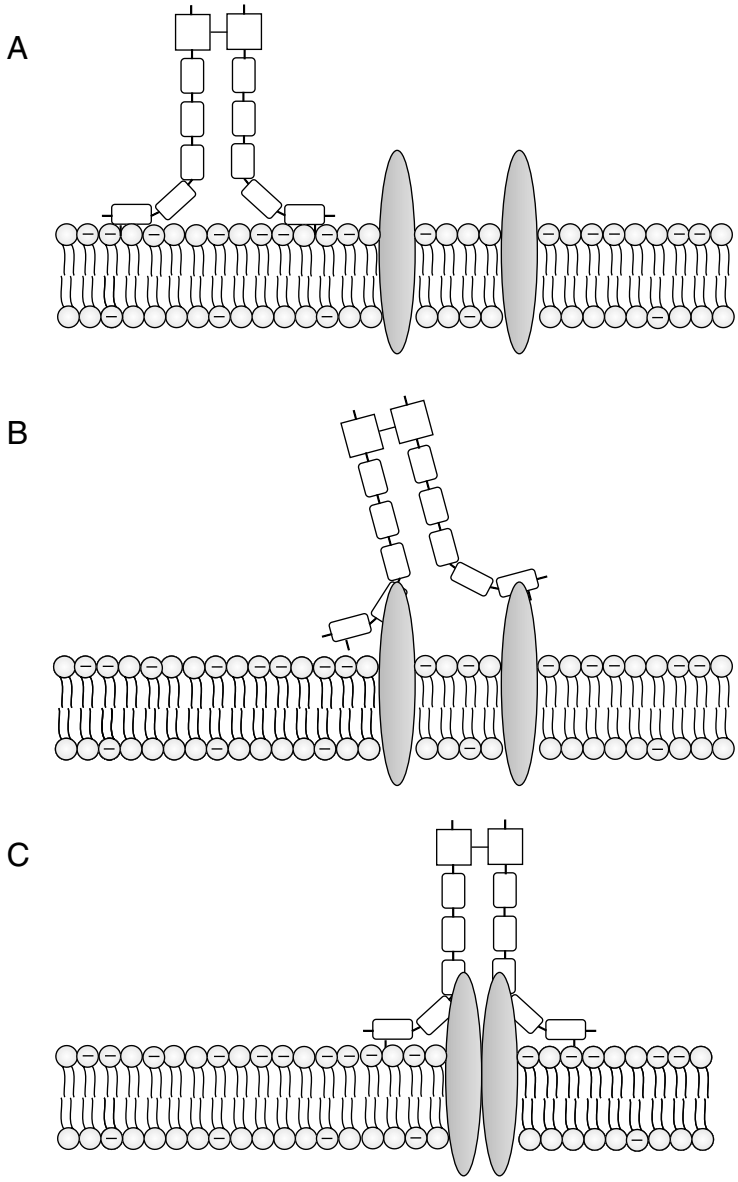

FIG.2. **Model for the mechanism by which β_2GPI-anti-β_2GPI antibody complexes can cause thrombosis.** The β_2GPI-anti-β_2GPI antibody complexes bind to the negatively charged phospholipids exposed on the platelet membrane (*A*). This is followed by the binding of these complexes to a receptor of the 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 β_2GPI-anti-β_2GPI 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: $\alpha_{IIb}\beta_3$ [30]. The affinity of prothrombin for this receptor is low compared to the affinity of fibrinogen and von Willebrand factor (vWF) for $\alpha_{IIb}\beta_3$. 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 $\alpha_{IIb}\beta_3$. $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$ 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* [33]. However, it also has been noted that infusion of APC in mice immunized with antiphospholipid antibodies significantly decreases thrombus size after injury [34].

Activation of the complement system may also play a role in the mechanism by which these antibodies exert their prothrombitic 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 β_2GPI-cardiolipin complexes [35], thus the results described are not easy to understand.

Thus, at present three possible mechanisms for the development of thrombosis in patients with anti-β_2GPI 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 β_2GPI-anti-β_2GPI 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 β_2GPI and the majority of the studies did not test if these antibodies recognize murine β_2GPI. 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 β_2GPI-anti-β_2GPI antibody complexes. In a hamster thrombosis model, in which it was shown that the anti-β_2GPI antibodies used recognize hamster β_2GPI, 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 β_2GPI-anti-β_2GPI antibody complexes [26]. 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 *in vivo* animal models. It is possible that all mechanisms play a role *in vivo* under different conditions. Furthermore, binding of the β_2GPI-anti-β_2GPI antibody complexes to negatively charged phospholipids, which is, as was shown in chapter 4, essential for activation of platelets, may *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.

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 β_2GPI-anti-β_2GPI antibody complexes stimulate the development of thrombosis *in vivo* similar to the mechanisms concluded from *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 [36]. *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 *in vitro* model. It has also been suggested that heparin may act as an immunomodulatory agent, which affects cytokine production and cell signalling [37]. Two possible mechanisms by which heparin inhibits the effects of β_2GPI-anti-β_2GPI 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 β_2GPI. Second, heparin may inhibit the binding of β_2GPI-anti-β_2GPI antibody complexes to a receptor of the LDL-receptor family. Both these mechanisms will result in abolishment of the effects of β_2GPI-anti-β_2GPI antibody complexes. We have shown in chapter 7 that heparin indeed inhibits the binding of dimeric β_2GPI 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 β_2GPI. 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 β_2GPI-anti-β_2GPI 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 β_2GPI-$F(ab)_2$ 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 β_2GPI in these patients. Recently, it has been reported that the majority of the anti-β_2GPI antibodies in patients with the antiphospholipid syndrome are directed against domain I of β_2GPI [38,39]. This knowledge was used to make a polyvalent construct that might induce tolerance of anti-β_2GPI secreting B-cells. To do so, four molecules of domain 1 of β_2GPI 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 β_2GPI gave inhibition of the antibody response to β_2GPI 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-β_2GPI antibodies or is inhibition of the antibody response just a decrease in circulating anti-β_2GPI antibodies caused by the clearance of complexes between anti-β_2GPI 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 β_2GPI-anti-β_2GPI 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

1. Horbach DA, Van Oort E, Donders RCJM, Derksen RHWM, De Groot PG. Lupus anticoagulant is the strongest risk factor for both venous and arterial thrombosis in patients with systemic lupus erythematosus - Comparison between different assays for the detection of antiphospholipid antibodies. *Thromb Haemost* 1996; 76: 916-924.
2. D'Angelo A, Safa O, Crippa L, Garlando A, Sabbadini MG, D'Angelo SV. Relationship of lupus anticoagulant, anticardiolipin, anti-β_2-GPI and anti-prothrombin autoantibodies with history of thrombosis in patients with the clinical suspicion of APA-syndrome. *Thromb Haemost* 1997; 78: 967-968.
3. Wahl DG, Guillemin F, de Maistre E, Perret C, Lecompte T, Thibaut G. Risk for venous thrombosis related to antiphospholipid antibodies in systemic lupus erythematosus-a meta-analysis. *Lupus* 1997; 6: 467-473.
4. Wahl DG, Guillemin F, de Maistre E, Perret-Guillaume C, Lecompte T, Thibaut G. Meta-analysis of the risk of venous thrombosis in individuals with antiphospholipid antibodies without underlying autoimmune disease or previous thrombosis. *Lupus* 1998; 7: 15-22.
5. Galli M, Luciani D, Bertolini G, Barbui T. Lupus anticoagulants are stronger risk factors of thrombosis than anticardiolipin antibodies inthe antiphospholipid: a systematic review of the literature. *Blood* 2002 [epub ahead of print].
6. Willems GM, Janssen MP, Pelsers MAL, Comfurius P, Galli M, Zwaal RFA, Bevers EM. Role of divalency in the high-affinity binding of anticardiolipin antibody-β_2-glycoprotein I complexes to lipid membranes. *Biochemistry* 1996; 35: 13833-13842.
7. Arnout J, Wittevrongel C, Vanrusselt M, Hoylaerts M, Vermylen J. Beta-2-glycoprotein I dependent lupus anticoagulants form stable bivalent antibody beta-2-glycoprotein I complexes on phospholipid surfaces. *Thromb Haemost* 1998; 79: 79-86.
8. Sheng Y, Kandiah DA, Krilis SA. Anti-β_2-glycoprotein I autoantibodies from patients with the "antiphospholipid" syndrome bind to β_2-glycoprotein I with low affinity: dimerization of β_2-glycoprotein I induces a significant increase in anti-β_2-glycoprotein I antibody affinity. *J Immunol* 1998; 161: 2038-2043.
9. Wagenknecht DR, McIntyre JA. Changes in β_2-glycoprotein I antigenicity induced by phospholipid binding. *Thromb Haemost* 1993; 69: 361-365.
10. Ichikawa K, Khamashta MA, Koike T, Matsuura E, Hughes GRV. β_2-Glycoprotein I reactivity of monoclonal anticardiolipin antibodies from patients with the antiphospholipid syndrome. *Arthritis Rheum* 1994; 37: 1453-1461.
11. Pengo V, Biasiolo A, Fior MG. Autoimmune antiphospholipid antibodies are directed against a cryptic epitope expressed when β2-glycoprotein I is bound to a suitable surface. *Thromb Haemost* 1995; 73: 29-34.
12. Chamley LW, Duncalf AM, Konarkowska B, Mitchell MD, Johnson PM. Conformationally altered β2-glycoprotein I is the antigen for anti-cardiolipin autoantibodies. *Clin Exp Immunol* 1999; 115: 571-576.
13. Galazka M, Tang M, DeBari VA, Kohles JD, Lee JK, Keil LB, Petersheim M. Modification of β2-glycoprotein I by glutardialdehyde. Conformational changes and aggregation accompany exposure of cryptic autoepitope. *Appl Biochem Biotech* 1999; 76: 1-13.

14. Wang S-X, Sun Y-T, Sui S-F. Membrane-induced conformational change in human apolipoprotein H. *Biochem J* 2000; 348: 103-106.
15. Regnault V, de Maistre E, Wahl D, Lecompte T. Monovalent binding of autoantibodies to β_2-glycoprotein I, detected using surface plasmon resonance at low antigen density. *Br J Haematol* 2000; 109: 187-194.
16. Hammel M, Kriechbaum M, Gries A, Kostner GM, Laggner P, Prassl R. Solution structure of human and bovine β_2-glycoprotein I revealed by small-angle X-ray scattering. *J Mol Biol* 2002; 321: 85-97.
17. Meroni PL, Raschi E, Camera M, Testoni C, Nicoletti F, Tincani A, Khamashta MA, Balestrieri G, Tremoli A, Hess DC. Endothelial activation by aPL: A potential pathogenic mechanism for clinical manifestations of the syndrome. *J Autoimmun* 2000; 15: 237-240.
18. Carreras LO, Machin SJ, Deman R, Defreyn G, Vermylen J, Spitz B, Van Assche A. Arterial thrombosis, intrauterine death and "lupus" anticoagulant: detection of immunoglobulin interfering with prostacyclin formation. *Lancet* 1981; 1: 244-246.
19. Carreras LO, Forastiero R, Martinuzzo M. Interaction between antiphospholipid antibodies and eicosanoids. In: Khamashta MA, ed. *The Hughes Syndrome.* London: Springer-Verlag; 2000:337-347.
20. Martinuzzo M, Maclouf J, Carreras LO, Lé vy-Toledano S. Antiphospholipid antibodies enhance thrombin-induced platelet activation and thromboxane formation. *Thromb Haemost* 1993; 70: 667-671.
21. Escolar G, Reverter JC, Lopez-Soto A, Garrido M, Cervera R, Ingelmo M, Castillo R, Ordinas A. Plasma from systemic lupus erythematosus patient with antiphospholipid antibodies promotes platelet aggregation. *Arterioscler Thromb* 1992; 12: 196-200.
22. Lin Y-L, Wang C-T. Activation of human platelets by the rabbit anticardiolipin antibodies. *Blood* 1992; 80: 3135-3143.
23. Arvieux J, Roussel B, Pouzol P, Colomb MG. Platelet activating properties of murine monoclonal antibodies to β_2-glycoprotein I. *Thromb Haemost* 1993; 70: 336-341.
24. Dunoyer-Geidre S, Kruithof EKO, Galve-de Rochemonteix B, Rosnoblet C, Gruenberg J, Reber G, de Moerloose P. Localization of β_2-glycoprotein I in late endosomes of human endothelial cells. *Thromb Haemost* 2001; 85: 903-907.
25. Arnout J. The pathogenesis of the antiphospholipid syndrome: a hypothesis based on parallelism with heparin-induced thrombocytopenia. *Thromb Haemost* 1996; 75: 536-541.
26. Jankowski M, Vreys I, Wittevrongel C, Boon D, Vermylen J, Hoylaerts MF, Arnout J. Thrombogenecity of beta2-glycoprotein I dependent antiphospholipid antibodies in a photochemically induced thrombosis model in the hamster. *Blood* 2002 [epub ahead of print].
27. Van Heerde WL, Sakariassen KS, Hemker HC, Sixma JJ, De Groot PhG. Annexin V inhibits the procoagulant activity of matrices of TNF-stimulated endothelium under blood flow conditions. *Arterioscler Thromb* 1994; 14: 824-830.
28. Moestrup SK, Schousboe I, Jacobsen C, Leheste JR, Christensen EI, Willnow TE. β_2-glycoprotein I (apolipoprotein H) and β_2-glycoprotein I-phospholipid complex harbor a recognition site for the endocytic receptor megalin. *J Clin Invest* 1998; 102: 902-909.

29. Lin KY, Pan JP, Yang DL, Huang KT, Chang MS, Ding PYA, Chiang AN. Evidence for inhibition of low density lipoprotein oxidation and cholesterol accumulation by apolipoprotein H (β_2-glycoprotein I). *Life Sci* 2001; 69: 707-719.
30. Byzova TV, Plow EF. Networking in the hemostatic system. Integrin $\alpha_{IIb}\beta_3$ binds prothrombin and influences its activation. *J Biol Chem* 1997; 272: 27183-27188.
31. Griffin JH. Blood coagulation - The thrombin paradox. *Nature* 1995; 378: 337-338.
32. Gruber A, Griffin JH. Direct detection of activated protein C in blood from human subjects. *Blood* 1992; 79: 2340-2348.
33. Simmelink MJ, Fernandez JA, Derksen RH, Griffin JH, De Groot PG. Low levels of activated protein C in patients with systemic lupus erythematosus do not relate to lupus anticoagulants but to low levels of factor II. *Br J Haematol* 2002; 117: 676-684.
34. Pierangeli SS, Liu X, Espinola RG, Harris EN. APL affects protein C activation in vivo. *Lupus* 11: 584. 2002. Abstract
35. Holers VM, Girardi G, Mo L, Guthridge JM, Molina H, Pierangeli SS, Espinola R, Xiaowei LE, Mao D, Vialpando CG, Salmon JE. Complement C3 activation is required for antiphospholipid antibody-induced fetal loss. *J Exp Med* 2002; 195: 211-220.
36. Erkan D. The relation between antiphospholipid syndrome-related pregnancy morbidity and non-gravid vascular thrombosis: A review of the literature and management strategies. *Curr Rheumatol Rep* 2002; 4: 379-386.
37. Rai R. aPL and pregnancy loss – beyond thrombosis. *Lupus* 11: 563. 2002. Abstract
38. Iverson GM, Victoria EJ, Marquis DM. Anti-β2 glycoprotein I (β2GPI) autoantibodies recognize an epitope on the first domain of b2GPI. *Proc Natl Acad Sci USA* 1998; 95: 15542-15546.
39. McNeeley PA, Dlott JS, Furie RA, Jack RM, Ortel TL, Triplett DA, Victoria EJ, Linnik MD. β_2-glycoprotein I-dependent anticardiolipin antibodies preferentially bind the amino terminal domain of β_2-glycoprotein I. *Thromb Haemost* 2001; 86: 595.
40. Linnik MD. A novel therapeutic approach for treating antiphospholipid syndrome based on tolerizing anti-β2-GPI B cells. *Stroke* 32: 330. 2001. Abstract

Nederlandse samenvatting

Inleiding

Het antifosfolipiden syndroom

Normaal gesproken worden lichaamsvreemde stoffen, zoals bacteriën en virussen, in het lichaam door het afweersysteem opgeruimd. Hiervoor worden afweerstoffen (ook wel antistoffen genoemd) gemaakt tegen deze lichaamsvreemde stoffen. Deze antistoffen herkennen een specifiek deel van de lichaamsvreemde stof, wat uiteindelijk als resultaat heeft dat deze lichaamsvreemde stof wordt opgeruimd. Soms ontspoort de productie van antistoffen, waardoor niet alleen antistoffen worden gemaakt tegen lichaamsvreemde stoffen, maar ook tegen lichaamseigen stoffen. Deze antistoffen worden autoantistoffen genoemd.

Antifosfolipiden antistoffen (aPL) zin een typisch voorbeeld van autoantistoffen. De aanwezigheid van deze antistoffen in bloed geeft een hogere kans op trombose (verstopping van een bloedvat ten gevolge van een bloedprop) en miskramen. Deze miskramen worden waarschijnlijk veroorzaakt door trombose van de bloedvaten in de moederkoek, waardoor de ongeboren vrucht niet genoeg voedingsstoffen op kan nemen om in leven te blijven. Het verband tussen de aanwezigheid van aPL en het voorkomen van trombose en miskramen wordt ook wel het antifosfolipiden syndroom genoemd.

Tot 1990 werd gedacht dat aPL gericht waren tegen fosfolipiden, zoals de naam aPL ook doet vermoeden. Fosfolipiden zijn een soort vetten en zijn belangrijke bouwstenen van de membraan (het omhulsel) die om elke cel in het lichaam zit. In 1990 werd ontdekt dat aPL niet gericht zijn tegen fosfolipiden, maar tegen eiwitten die aan fosfolipiden kunnen binden. De belangrijkste eiwitten waartegen aPL gericht kunnen zijn, zijn β_2-glycoproteine I (β_2GPI) en protrombine. Over de functie van β_2GPI in het lichaam is weinig bekend. Personen die dit eiwit missen door een fout in het DNA (het genetische materiaal) lijken hierdoor geen problemen te hebben. Protrombine is een belangrijk eiwit in de bloedstolling. Protrombine wordt dan geknipt wat resulteert in de vorming van trombine (zie bloedstelping). aPL die gericht zijn tegen β_2GPI en protrombine worden ook wel anti-β_2GPI en anti-protrombine antistoffen genoemd.

aPL kunnen op twee manieren worden opgespoord: (1) door de binding van de antistoffen aan β_2GPI en protrombine (die eventueel gebonden zijn aan fosfolipiden) te meten en (2) door lupus anticoagulante (LAC) activiteit te meten. LAC activiteit wordt bepaald in plasma (dat deel van het bloed wat overblijft na de verwijdering van alle bloedcellen) met behulp van stoltesten. Met een stoltest kan worden bepaald hoe snel (of hoe langzaam) een plasma stolt. Antistoffen met LAC activiteit zorgen ervoor dat plasma langzamer stolt, wat normaal gesproken bloeding betekent. Dit is dus het tegenovergestelde van de trombose die in de patiënt wordt gezien. Niet alle aPL bezitten LAC activiteit. Gezien het feit dat met LAC activiteit een functie, en dus niet alleen de aanwezigheid, van antistoffen wordt getest, worden antistoffen met LAC activiteit ook wel ‘functionele’ antistoffen genoemd. Ook is LAC activiteit sterker met trombose geassocieerd in patiënten dan alleen de aanwezigheid van antistoffen.

Bloedstelping

Bloedstelping is het proces wat ervoor zorgt dat bij een beschadiging van de bloedvatwand de wond wordt hersteld zonder dat er onnodig bloedverlies optreedt. Dit proces is

onder normale omstandigheden strikt gereguleerd, waardoor alleen een stolsel kan ontstaan waar een bloedvat is beschadigd. Dit stolsel sluit dus niet de bloedstroom af. Wordt deze balans echter verstoort, dan kan in het bloedvat een trombus ontstaan die wel de bloedstroom afsluit.

Op het moment dat er een wond ontstaat, treden er een aantal aan elkaar gekoppelde reacties op. Allereerst trekt het bloedvat samen, waardoor de hoeveelheid bloed die door het vat stroomt wordt verminderd. Door de beschadiging van de bloedvatwand worden eiwitten (zoals collageen en tissue factor) die normaal niet in contact komen met het bloed blootgesteld aan de bloedstroom. Dit heeft tot gevolg dat bloedplaatjes, die in rustende toestand circuleren in het bloed, hechten aan een aantal van deze eiwitten (bv. collageen). Hierdoor worden de plaatjes geactiveerd, waardoor ze weer andere bloedplaatjes kunnen binden. Op deze manier ontstaat een bloedplaatjesprop die de wond afsluit. Deze prop is alleen niet erg stevig, waardoor hij weg gespoeld kan worden door de bloedstroom in het vat. Daarom wordt de prop verstevigd m.b.v. een fibrinenetwerk. De eerste aanzet tot de vorming van dit fibrinenetwerk is de binding van actief factor VII, wat in kleine hoeveelheden in het bloed circuleert, aan tissue factor, een eiwit dat door de vaatwandbeschadiging wordt blootgesteld aan de bloedstroom. Hierop wordt een waterval van elkaar opvolgende reacties aangezet (de stollingscascade), die uiteindelijk resulteren in de vorming van trombine (zie hoofdstuk 1, figuur 3). Trombine knipt dan fibrinogeen tot fibrine, wat onoplosbaar is. Ook activeert trombine factor XIII, die het fibrinenetwerk verstevigt. Het resultaat hiervan is welbekende het korstje op de wond. Na het genezen van de wond moet het stolsel ook weer afgebroken worden. Dit gebeurt in een proces dat fibrinolyse wordt genoemd. Voor de meeste van de reacties in de stollingscascade is de binding van stolfactoren aan negatief geladen fosfolipiden nodig. Onder normale omstandigheden zijn alleen fosfolipiden zonder lading aanwezig op de buitenkant van de membraan van de cellen, maar door activatie van bv. bloedplaatjes komen er ook negatief geladen fosfolipiden op de membraan van deze cellen.

Om te voorkomen dat de het proces wat zorgt voor de afsluiting van de wond uit de hand loopt (en er dus een te trombus ontstaat die niet alleen de wond, maar ook het vat afsluit) zijn er een aantal remmers van de bloedstolling in het bloed aanwezig. Ook worden er in stollingscascade eiwitten (proteine C) geactiveerd die deze cascade remmen, waardoor deze zichzelf in de hand kan houden. Een ander belangrijk mechanisme om dit proces te reguleren is dat het proces wordt gestart door eiwitten die onder normale omstandigheden niet in contact komen met het bloed. Treedt er echter een beschadiging op van de vaatwand, dan kunnen deze eiwitten wel in contact komen met bloed, waardoor de bloedstelping gestart wordt.

β_2GPI en anti-β_2GPI antistoffen

Als anti-β_2GPI antistoffen aan β_2GPI binden heeft dit als resultaat dat dit complex veel sterker aan negatief geladen fosfolipiden bindt dan β_2GPI zelf. Hierdoor is ook de LAC activiteit te verklaren. Zoals al eerder genoemd, zijn negatief geladen fosfolipiden nodig om de reacties van de stollingscascade goed te kunnen laten verlopen. In een stoltest worden deze negatief geladen fosfolipiden toegevoegd op het moment dat de reactie wordt gestart met een activator van de stollingscascade. Als er nu anti-β_2GPI antistoffen in dit plasma aanwezig zijn, kunnen deze aan β_2GPI (wat ook in plasma aanwezig is) binden. Dit complex

bindt dan weer aan de negatief geladen fosfolipiden, waardoor deze fosfolipiden niet meer in staat zijn om stolfactoren te binden. Hierdoor verloopt de stollingscascade minder snel en is dus ook tijd voordat het plasma is gestold langer. Dit is dus LAC activiteit.

Anti-β_2GPI antistoffen die β_2GPI hebben gebonden (β_2GPI-anti-β_2GPI antistof complexen) kunnen op een aantal manieren met de bloedstelping interfereren. De belangrijkste van deze manieren lijken te zijn: (1) de activatie van cellen die betrokken zijn bij de bloedstelping en (2) remming van de activatie van protei022ne C, waardoor de stollingscascade minder goed wordt geremd. Wij hebben ons geconcentreerd op het eerste mechanisme en dan de activatie van bloedplaatjes. Hiervoor hebben we een model gebruikt wat de bloedstroom in een bloedvat met een wond nabootst en dat model wordt hieronder uitgelegd.

Het perfusiesysteem

Met het perfusiesysteem kan naar de binding van bloedplaatjes aan verschillende oppervlakken en met verschillende stromingssnelheden, representatief voor de stroomsnelheid van het bloed in aders (venen) en slagaders (arteriën), gekeken worden. Hiervoor wordt bloed van vrijwilligers gebruikt waaraan een stof is toegevoegd die activatie van de stollingscascade tegengaat. Dit bloed wordt met een gedefinieerde snelheid door slangetjes, die verbonden zijn met de perfusiekamer, getrokken. De perfusiekamer (hoofdstuk 1, figuur 4) bevat een dekglaasje waarop bv. collageen gecoat is. Op deze manier wordt dus alleen het stukje van de vorming van de bloedplaatjesprop tijdens de bloedstelping eruit gelicht om beter kunnen bestuderen. Na de perfusie worden de bloedplaatjes op het dekglaasje gekleurd en wordt de bedekking van het dekglaasje gemeten met behulp van een microscoop.

Receptoren

Een goede verklaring voor de effecten van β_2GPI-anti-β_2GPI antistof complexen op cellen, zou kunnen zijn dat deze complexen aan receptoren (een soort antennes) op de buitenkant van deze cellen binden. Goede kandidaten hiervoor zijn leden van een familie van deze antennes die de LDL-receptor familie wordt genoemd. LDL staat voor low-density lipoprotein en wordt ook wel het slechte cholesterol genoemd. Van een van deze leden, megaline, is bekend dat β_2GPI er aan kan binden. Megaline zit op cellen in de nieren en zal dus niet zo'n grote rol spelen in het antifosfolipiden syndroom, maar bv. LRP (LDL-Receptor gerelateerd Proteine) wordt gevonden op cellen in de moederkoek en LRP8 zit als antenne op bloedplaatjes. Deze zouden dus wel een rol kunnen spelen in de ontwikkeling van trombose in het antifosfolipiden syndroom.

Doel van de studie

Het doel van deze studie is te onderzoeken wat het mechanisme is waardoor mensen met aPL (en in het bijzonder anti-β_2GPI antistoffen) trombose ontwikkelen.

Resultaten

Dimerizatie van β_2GPI door anti-β_2GPI antistoffen

Er zijn twee mechanismen beschreven voor manier waarop anti-β_2GPI antistoffen aan β_2GPI binden: (1) een anti-β_2GPI antistof heeft twee pootjes en bindt met ieder pootje een

β_2GPI (dimerizatie) en (2) een anti-β_2GPI antistof bindt een β_2GPI, waardoor er een vormverandering ontstaat in β_2GPI. Beide mechanismen zorgen ervoor dat het β_2GPI-anti-β_2GPI antistof complex veel beter aan fosfolipiden kan binden dan β_2GPI zelf. Een schematische tekening van deze mechanismen is te vinden in hoofdstuk 8 (figuur 1). Wij hebben ons geconcentreerd op het eerste mechanisme. Hiervoor hebben we een eiwit gemaakt wat bestaat uit 2 keer β_2GPI en twee keer apple4. Dit is een deel van stolfactor XI wat zichzelf spontaan bindt. Hierdoor ontstaat dus ook een dimeer van β_2GPI (voor een schematische tekening zie hoofdstuk 3, figuur 1A). In hoofdstuk 3 hebben we laten zien dat dit dimeer β_2GPI inderdaad veel beter aan fosfolipiden bindt dan β_2GPI zelf en dat het in staat is de stoltijd van plasma te verlengen (en dus LAC activiteit bezit). Met dit dimeer β_2GPI kunnen dus de verdere effecten van β_2GPI-anti-β_2GPI antistof complexen op de verschillende componenten die een rol spelen in de bloedstelping getest worden.

De effecten van dimeer β_2GPI, anti-β_2GPI en anti-protrombine antistoffen op plaatjesactivatie

Met het perfusiesysteem zijn de effecten van dimeer β_2GPI en anti-β_2GPI op de binding (adhesie) van bloedplaatjes aan collageen getest (hoofdstuk 4). Dimeer β_2GPI en de meeste LAC positieve anti-β_2GPI antistoffen verhoogden de adhesie van bloedplaatjes aan collageen. Ook hebben we laten zien dat de aggregaten (klontjes van bloedplaatjes) groter zijn in aanwezigheid van dimeer β_2GPI dan in afwezigheid van dimeer β_2GPI.

Een LAC positieve anti-β_2GPI antistof die de adhesie van bloedplaatjes aan collageen niet stimuleerde is 4F3. Om te kijken of deze antistof misschien de binding van dimeer β_2GPI (of β_2GPI-anti-β_2GPI antistof complexen) aan een mogelijke receptor op het bloedplaatje remt, werd 4F3 samen met dimeer β_2GPI aan een perfusie toegevoegd. De verhoogde adhesie van bloedplaatjes in aanwezigheid van dimeer β_2GPI verdween hierdoor. Dit betekent dat 4F3 inderdaad de binding van dimeer β_2GPI aan een receptor op het bloedplaatje remt. Om te kijken of receptoren van de LDL-receptor familie (zoals LRP8) een rol spelen, werd een perfusie uitgevoerd in aanwezigheid van dimeer β_2GPI (of anti-β_2GPI antistoffen) en RAP. RAP is een eiwit wat aan alle (tot dusver) bekende leden van de LDL-receptor familie zo sterk kan binden, dat andere eiwitten die aan deze receptoren kunnen binden daar geen mogelijkheid meer voor hebben. In aanwezigheid van RAP verdween inderdaad de verhoogde adhesie van bloedplaatje die we zagen met alleen dimeer β_2GPI. Dit betekent dat er een sterke aanwijzing is dat een receptor van de LDL-receptor familie een rol speelt in de versterkte activatie van bloedplaatjes door dimeer β_2GPI (of anti-β_2GPI antistoffen).

Een andere aanwijzing voor het mechanisme waardoor β_2GPI-anti-β_2GPI antistof complexen effecten hebben op bloedplaatjes kwam door het gebruik van andere oppervlakken, zoals fibrinogeen. In aanwezigheid van dimeer β_2GPI werd hier geen verhoogde adhesie van bloedplaatjes gevonden. Een verklaring hiervoor is dat bloedplaatjes door aan collageen te hechten zover geactiveerd worden dat ze negatief geladen fosfolipiden op hun oppervlak krijgen. Binding van bloedplaatjes aan fibrinogeen geeft een veel minder grote activatie van bloedplaatjes en er worden dan ook geen negatief geladen fosfolipiden gevonden op het oppervlak van bloedplaatjes die gebonden hebben aan fibrinogeen. Zoals al eerder genoemd binden dimeer β_2GPI en β_2GPI-anti-β_2GPI antistof complexen erg sterk aan deze negatief geladen fosfolipiden. Daarom denken wij dat het volgende mechanisme een verklaring kan zijn voor de effecten van β_2GPI-anti-β_2GPI antistof complexen op bloedplaatjes. Allereerst

binden β_2GPI-anti-β_2GPI antistof complexen aan de negatief geladen fosfolipiden op het oppervlak van geactiveerde bloedplaatjes. Dit wordt gevolgd door binding van de complexen aan een receptor van de LDL-receptor familie, die dan in staat is te dimerizeren. Door deze dimerizatie, worden signalen gestart waardoor het bloedplaatje nog sterker geactiveerd wordt. Dit resulteert uiteindelijk in de vorming van een prop, die i.p.v. alleen de wond, het hele vat afsluit (voor een schematische tekening zie hoofdstuk 8, figuur 2). Dit mechanisme geldt alleen voor slagaders, omdat de stroomsnelheid van het bloed in de experimenten overeenkomt met de stroomsnelheid van het bloed in slagaders.

In tegenstelling tot anti-β_2GPI antistoffen, hebben anti-protrombine antistoffen geen effect op de adhesie van bloedplaatjes aan collageen. Een verklaring hiervoor is dat anti-protrombine antistoffen niet (en anti-β_2GPI antistoffen wel) aan bloedplaatjes kunnen binden.

Effecten van dimeer β_2GPI en anti-β_2GPI antistoffen op de aggregatie van bloedplaatjes

Naast het binden van bloedplaatjes aan bv. collageen, vormen de bloedplaatjes ook een prop (aggregaat) door aan elkaar te binden. Dit wordt niet in stromend bloed getest, maar in een buisje met plasma en bloedplaatjes. Hieraan wordt, al roerende, een stof aan toegevoegd die bloedplaatjes kan activeren (bv. ADP). De effecten van dimeer β_2GPI en anti-β_2GPI antistoffen op bloedplaatjes aggregatie zijn met behulp van dit systeem getest. Dimeer β_2GPI had geen enkel effect op de aggregatie van bloedplaatjes na activatie met ADP. In tegenstelling tot dimeer β_2GPI hadden anti-β_2GPI antistoffen wel een stimulerend effect op de aggregatie van bloedplaatjes in aanwezigheid van ADP (hoofdstuk 6). Omdat dit de eerste observatie was dat de effecten van dimeer β_2GPI en anti-β_2GPI antistoffen verschilden, zijn vervolgexperimenten gedaan. Hierin is aangetoond dat het een anti-β_2GPI antistof was die de bloedplaatjes activeerde, maar dat het een klomp (een aggregaat) van anti-β_2GPI antistoffen was, wat de bloedplaatjes activeerde. Dit geeft een aspecifieke activatie van de bloedplaatjes, die niet veroorzaakt kan worden door dimeer β_2GPI.

Binding van dimeer β_2GPI aan receptoren van de LDL-receptor familie

In hoofdstuk 4 hebben we aangetoond dat dimeer β_2GPI en anti-β_2GPI antistoffen waarschijnlijk bloedplaatjes activeren via een mechanisme waar een receptor van de LDL-receptor familie bij is betrokken. Daarom hebben we met behulp van Biacore analyse (een apparaat wat binding meet) de binding van dimeer β_2GPI aan LRP en megaline gemeten. Beide eiwitten binden inderdaad dimeer β_2GPI. Bij de binding van dimeer β_2GPI aan LRP zijn een stuk van β_2GPI wat op de grens van domein 1 en 2 ligt en domein 5 van β_2GPI betrokken (voor een schematische tekening met de domeinen van β_2GPI zie hoofdstuk 2, figuur 2).

Conclusie

Met het gedane onderzoek hebben we aangetoond dat dimeer β_2GPI op een zelfde manier als anti-β_2GPI antistoffen werkt. Beiden zijn in staat om bloedplaatjes te activeren via een mechanisme waarbij negatief geladen fosfolipiden en een receptor van de LDL-receptor familie betrokken zijn. Met deze bevindingen heeft een link gelegd tussen de LAC activiteit die wordt gemeten in het lab en de trombose die gezien wordt in patiënten.

Dankwoord

Het ei is gelegd, alles is af! Vier jaar proefjes doen en schrijven hebben uiteindelijk geresulteerd in dit boekje. Zonder goede begeleiding, vriendschap en gezelligheid (in het lab, maar vooral ook daarbuiten) was dit niet zover gekomen, daarom wil ik graag een aantal mensen bedanken.

Mijn promotoren Prof. Dr. Bijlsma en Prof Dr. De Groot en mijn co-promotor Dr. Derksen. *Hans*, ook al was je niet als directe begeleider bij het onderzoek betrokken, je was altijd geï nteresseerd.
Flip, vier jaar geleden hadden we een geheel andere inhoud van dit boekje in gedachten. Helaas deden de muizen niet wat wij wilden dat ze deden. Toch is het allemaal gelukt, en daar hebben jouw enorme optimisme en je ‘ open deur’ begeleiding in grote rol in gespeeld.
Ron, geweldig hoe jij altijd enthousiast kunt zijn over proeven. Je klinische kijk op het onderzoek en alle patiënt materialen waren onmisbaar.

Joost Meijers, jouw idee (of was het toch Flip’s idee?) is toch wel het fundament waarop dit onderzoek is gebouwd.

Peter Lenting, zonder jouw hulp was hoofdstuk 7 er niet geweest. Bedankt voor het uitleggen van alle ins en outs van de Biacore en de stoomcursus ‘ik ga op reis’.

Jef Arnout, bedankt voor alle monoclonale antistoffen.

Winnie, het was gezellig samen! Bedankt voor alle zuiveringen en het betere knip- en plakwerk.

Marco en Monique, studenten. *Marco*, ondanks dat jouw bijdrage aan het onderzoek niet in het boekje staat, was die wel waardevol. Hopelijk wordt het nog wat met LRP8. *Monique*, je proeven met de BIAcore hebben geleid tot hoofdstuk 7!

De APS groep *Bas, Maarten, Menno en Rolf*, veel succes met jullie onderzoek. Jullie komen er wel! *Marleen*, mede-APS AIO, je hebt me wegwijs gemaakt in de wereld van zuiveringen en de KC10. Bedankt voor alle gezellige en vruchtbare werkbesprekingen.

Mede-labgenoten, het was gezellig. *Marion, Gertie, Martin en Erik*, bedankt voor het beantwoorden van al mijn vragen. *Suzanne*, bedankt voor de vele gezellig uurtjes en vergeet het niet: morgen is er weer een dag. *Patricia*, geef ze niet al te veel stof voor jouw promotie.

De routine, bedankt voor de gezellige uurtjes stollen (ik heb het al gemist) en alle hulp.

Medewerkers van het GDL, helaas is het niet gelukt met de muizen. Toch wil ik jullie graag bedanken voor alle zorg. En natuurlijk ook *Gerry, Mariska en Stefan*, bedankt voor de gezellige samenwerking.

Patiënten en minidonoren, bedankt voor alle donaties.

De dames van het secretariaat, bedankt voor jullie hulp met administratieve zaken.

Mieke & Wybo, Jasper & Denise en Martijn & Ingrid, bedankt voor alle gezellige uurtjes vol met lekker eten en humor. Mieke en Denise, bedankt dat jullie mijn paranimfen willen zijn.

Jan, Hélène, Dagmar en Kirsten, altijd stond de deur open voor een gezellig ontspannend bezoekje tussen de bedrijven door. Bedankt. Jan, de omslag is waanzinnig mooi.

Irma en Marc, rasechte limburgers. Nu heb ik weer tijd voor meer dan een bliksembezoek. Enne Irma, het perfusiesysteem is het helemaal!

Lieve pap en mam, bedankt dat jullie me altijd hebben laten doen wat ik graag wilde. Een bezoekje aan jullie deed wonderen in stress-situaties.

Lieve Rutger, bedankt dat je er altijd was al ik het weer eens niet zag zitten (en me dan ook nog zover kreeg dat ik het weer wel zag zitten). Ik hou van je (ook al heb je dan de voorkant niet gehaald)!

Curriculum Vitae

Bianca Lutters werd geboren op 18 januari 1976 te Weert. In 1994 behaalde zij het VWO diploma aan de Philips van Horne scholengemeenschap te Weert. In dat zelfde jaar werd aangevangen met de studie medische biologie aan de Universiteit van Utrecht. In 1999 werd het doctoraal diploma behaald met als hoofdvak Virologie (Dr. R.J. de Groot en Dr. J.D.F. Mijnes, Faculteit Diergeneeskunde, Universiteit Utrecht) en als bijvak Biochemie (Prof. Dr. I. Braakman en Dr. A. Benham, Faculteit Geneeskunde, Universiteit van Amsterdam). Van februari 1999 tot februari 2003 was de schrijfster van dit proefschrift werkzaam als assistent in opleiding bij de vakgroep Haematologie van het Universitair Medisch Centrum in Utrecht onder begeleiding van Prof. Dr. Ph.G. de Groot, Prof. Dr. J.W.J. Bijlsma en Dr. R.H.W.M. Derksen. De resultaten van het onderzoek zijn beschreven in dit proefschrift.