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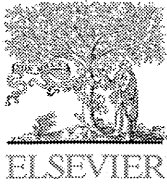

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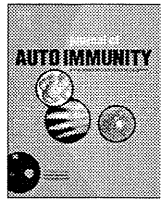

Factor XI is a substrate for oxidoreductases: Enhanced activation of reduced FXI and its role in antiphospholipid syndrome thrombosis

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ABSTRACT

Factor XI (FXI), a disulfide-linked covalent homodimer, circulates in plasma, and upon activation initiates the intrinsic/consolidation phase of coagulation. We present evidence that disulfide bonds in FXI are reduced to free thiols by oxidoreductases thioredoxin-1 (TRX-1) and protein disulfide isomerase (PDI). We identified that Cys362–Cys482 and Cys118–Cys147 disulfide bonds are reduced by TRX-1. The activation of TRX-1-treated FXI by thrombin, FXIIa or FXIa was significantly increased compared to non-reduced FXI, indicating that the reduced factor is more efficiently activated than the oxidized protein. Using a novel ELISA system, we compared the amount of reduced FXI in antiphospholipid syndrome (APS) thrombosis patients with levels in healthy controls, and found that APS patients have higher levels of reduced FXI. This may have implication for understanding the contribution of FXI to APS thrombosis, and the predisposition to thrombosis in patients with elevated plasma levels of reduced FXI.

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1. Introduction

The antiphospholipid syndrome (APS) is an autoimmune disorder characterized by the presence of auto-antibodies to the main auto-antigen β2-glycoprotein I (β2GPI) [1] in conjunction with either a venous and/or an arterial thrombotic event [2]. Most other thrombotic disorders affect either the venous circulation alone or the arterial circulation alone. Hence, an important goal in APS research has been to try and understand mechanistically why both sides of the circulation can be affected. It is relevant to consider studies that demonstrate that elevated levels of coagulation FXI are associated with an increased risk of both venous [3] and arterial thrombosis, especially cerebral arterial thrombosis [4], the same vascular beds affected in APS.

An avenue which we consider pertinent to explore with regards to; a) more fully understanding FXI regulation in APS, and b) the development of FXI assays which accurately predict APS thrombosis risk, is the study of post-translational redox modification of FXI. Our group has developed the proof of concept of this approach with work on the plasma protein β2GPI [5–8]. Insights from this area encouraged us to pursue this agenda with regards to FXI. β2GPI an abundant plasma protein, has been shown to bind and regulate FXI activation via the former's C-terminal domain [9,10]. The disulfide bond between Cys288 and Cys326 completes the formation of β2GPI's unique C-terminal loop. We have shown that thioredoxin reduces this disulfide bond in plasma [5,6]. The reduced form has distinct properties compared to oxidized β2GPI, it is able to protect endothelial cells against oxidative stress injury [6], and has increased affinity for binding von Willebrand factor [7]. β2GPI is

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the major autoantigen in the antiphospholipid syndrome (APS) [1], a disorder characterized by venous and arterial thrombosis [2]. We have developed an ELISA system which can measure the reduced form of β2GPI in human plasma and serum [6]. We utilized this assay to quantify the levels of reduced and oxidized forms of β2GPI in patients with APS compared to various control groups, and found significant differences [8].

It is emerging that mature protein function can be controlled by specialised disulfide bonds, known as allosteric disulfides [11–13]. Allosteric control is defined as a change in one site, the allosteric site, that influences another site by exploiting the protein's flexibility [14]. By definition, therefore, redox change in an allosteric disulfide influences another site in the protein. The influence at the other site can manifest as new ligand binding, such as protein dimerisation [15], or change in the activation of a zymogen, which is the situation described herein. A number of allosteric disulfides have been identified to date and there are several other potential examples at various stages of characterization [16,17]. A recent example of an allosteric disulfide in a thrombosis protein is the Cys2431-Cys2453 disulfide in von Willebrand Factor [18], which mediates lateral self-association of the protein.

Human FXI is the zymogen of the plasma serine protease FXIa that participates in the consolidation phase of intrinsic coagulation. Factor XI is a disulfide-linked homodimer consisting of two identical 80 kDa subunits, each containing four tandem repeat sequences designated apple (A1 to A4 from the N-terminus) domains and a C-terminal trypsin-like catalytic domain [19,20]. Cys321 within the A4 domain forms the interchain disulfide bond between the two subunits of the dimer. Each subunit can be cleaved by thrombin, FXIIa or FXIa at an internal single bond (Arg369-Ile370), leading to the generation of FXIa [19]. There are 19 disulfide bonds present in each of the subunits of human FXI in addition to the interchain disulfide bond [20]. However, the functional significance of these disulfide bonds has not been fully elucidated.

The present study was undertaken to determine whether thiol-disulfide exchange within the FXI molecule can be regulated by oxidoreductases such as TRX-1 or PDI, and to assess the functional significance of this modification for FXI activation by FXII, thrombin, and FXIa (autoactivation). We also quantitated the relative amount of reduced FXI in APS patients compared to levels obtained in normal controls.

2. Materials and methods

2.1. Chemicals and reagents

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), dithiothreitol (DTT), polyethylene glycol 8000 (PEG 8000) were purchased from Sigma (St Louis, MO). N-(3-maleimidylpropionyl) biocytin (MPB) and pre-cast NuPAGE 4–12% gradient SDS-PAGE gels were purchased from Invitrogen (Madison, WI). PolyScreen polyvinyldiethylene fluoride (PVDF) transfer membranes, Western blot chemiluminescence reagents and reflective autoradiography film were purchased from GE Healthcare (Bucks, UK). Argon from BOC gases (Sydney, NSW, Australia).

2.2. Proteins and plasma

Human recombinant TRX-1 was purchased from R&D Systems (Minneapolis, MN). Recombinant rat TRX-1 reductase (TRX-R) from American Diagnostica (Stamford, CT). Thrombin from human plasma, hirudin, human serum albumin (HSA), bovine serum albumin (BSA), 4-nitroaniline (pNA), recombinant human protein disulfide isomerase (PDI) was from Medical & Biological Laboratories Co. (Woburn, MA). Dextran sulfate (DS, M.W. 500,000), polybrene (Hexadimethrine Bromide), anti-goat/sheep IgG-ALP from Sigma (St. Louis, MI). Rabbit brain cephalin extract from Innovative Research (Novi, MI). Total human FXI antigen assay kit from Molecular Innovations (Novi, MI). Plasma derived FIX, FX, FXI, FXIa and FXI deficient plasma were purchased from Haematologic Technologies Inc (Essex Junction, VT). Human FXIIa from Merck KGaA (Darmstadt, Germany). Streptavidin-HRP and anti-goat HRP were purchased from Dako (Carpinteria, CA). Affinity purified goat IgG anti-human FXI monoclonal antibody from Affinity Biologicals (Hamilton, ON, Canada), Chromogenic substrate S2366, S2238 and S2302 were obtained from Chromogenix Instrumentation Laboratory SpA (Milano, Italy).

2.3. Generation and MPB labelling of free-thiols in FXI

All reagents were diluted in 20 mM HEPES buffer containing 0.14 M NaCl, 1.5 mM $CaCl_2$, 4 mM KCl, 0.5 mM Na_2HPO_4, pH 7.4 (HBS) which was based on the method described previously by our group for $β_2$GPI [5,6,8]. Detailed methodology of generation and MPB labelling of free-thiols in FXI is described in detail in supplementary information.

2.4. Mass spectrometry

In order to examine the specific cysteines targeted by TRX-1 in FXI, purified human FXI was treated with TRX-1/TRX-R/NADPH (TRN) and labelled with MPB as described in supplementary methods. Labelled FXI was subsequently run on an SDS-PAGE gel and stained with silver. The separated bands were excised, alkylated with iodoacetamide, digested with trypsin and analysed by LC–MS/MS. Detailed methods are presented in Supplementary Information.

2.5. Effect of reduced TRX-1/PDI on the activation of FXI

Activation of reduced TRX-1/PDI treated FXI by Thrombin/FXIIa in the presence of DS TRX-1/TRX-R/NADPH or PDI/DTT treated FXI (60 nM) was mixed with various concentrations of DS (0.125–1 ug/ml) in TBSA and incubated at 37 °C for 5 min, followed by addition of thrombin (2 nM) or FXIIa (2 nM) and incubated for a further 10 min at 37 °C. Reactions were stopped by placing on ice and diluting 1:5 with TBS. Hirudin (25 U/ml) and polybrene (200 ug/ml) were added to neutralize thrombin and DS. The generation of FXIa was determined by its ability to cleave S2366 (1.2 mM). Reaction speed at each time point was expressed as the increase of mOD at 405 nm per minute.

2.6. Time course of reduced TRX-1/PDI on the activation of FXI by thrombin

FXI (0.1 uM) was pre-incubated with TRX-1 (0.175 μM) activated with TRX-R (0.5 nM) + NADPH (10 μM) or PDI (25 nM) activated by DTT (2.5 uM) in HBS buffer at 37 °C under argon. At defined time points, the reaction mixture (FXI:60 nM) was aliquoted and incubated with DS (0.25 ug/ml) in TBSA (50 mM Tris–HCl/150 mM NaCl/0.1% BSA, pH 7.6) for 5 min at 37 °C. Thrombin (2 nM) was then added and incubated for a further 10 min at 37 °C. Reactions were stopped by hirudin (25 U/ml) and polybrene (200 μg/ml). 50 ul aliquots of each reaction mixture were dispensed into microtiter wells and mixed with 50 μl of S2366 (1.2 mM). The optical density was measured at 405 nm by using a Microplate Scanning Spectrophotometer. The amount of FXIa generated was derived from a standard curve constructed with known concentrations of FXIa.

2.7. Autoactivation of reduced TRX-1 treated FXI

The autoactivation of FXI in the presence of a negatively charged surface was investigated by incubating TRN or HSA-treated FXI (30 nM) with DS (0.25 ug/ml) in TBSA at 37 °C for 0–60 min. At specific time points, aliquots of the reaction were mixed with polybrene (200 ug/ml) and diluted 1:5 with TBS. The generation of FXIa was assayed by incubating with the chromogenic substrate S2366. The concentration of FXIa generated was derived from a standard curve plotted against known concentrations of FXIa.

2.8. Effect of TRN on the activities of thrombin, FXIIa and FXIa

2.8.1. Chromogenic assay of TRN treated thrombin/FXIIa/FXIa

To determine if the effect of TRN on the activation of FXI is partly mediated by the enhancement on the enzymatic activities of thrombin, FXIIa and/or FXIa, the capacities of these proteases treated by TRN or HSA to cleave their respective chromogenic substrates was assessed. Briefly, thrombin was incubated with TRX-1/TRX-R/NADPH mixture or HSA at 37 °C for 10 min. The molar ratio of TRX-1/HSA to thrombin was 52.5:1 which was identical to that in the TRX-1 reduced FXI activation reaction system. The kinetics of hydrolysis of S2238 (6.25–200 μM) by thrombin (0.5 nM) was examined at 37 °C in 0.1 M NaCl, 0.02 M Tris–HCl (pH 7.5) containing 0.1 mg/ml BSA, 0.1% PEG 8000, and 2.5 mM $CaCl_2$ (TBS/Ca^{2+}). The rate of hydrolysis was recorded at 405 nm. Michaelis–Menten kinetic parameters (K_m and V_{max}) were calculated using the average from three separate experiments. Values of V_{max} were converted to nM pNA/s using an extinction coefficient of 9800 optical density units at 405 nm per mole of pNA. Turnover number (K_{cat}) was calculated from the ratio of V_{max} to enzyme concentration. The kinetic assay of TRN-treated FXIIa or FXIa on the hydrolysis of their respective chromogenic substrate S2302 or S2366 were similar to that described above for FXI except that the reagents were diluted in TBSA and the final concentrations of FXIIa and S2302 were 5 nM and 15.625–1000 μM, respectively. For the FXIa chromogenic assay, the final concentrations of FXIa and S2366 were 2 nM and 0.25–4 mM, respectively.

2.9. Assay for in vivo detection of reduced FXI

2.9.1. Blood collection

Blood samples from patients with APS arterial/venous thrombosis ($n = 20$, M/F: 8:12, mean age: 50.4 ± 15.2 years) were used from an international collaborative collection involving three centers (University of New South Wales (Australia), University of Athens (Greece) and Hokkaido University School of Medicine (Japan)). Age and sex matched normal control samples ($n = 15$, M/F: 6:9, mean age: 53.7 ± 10.4 years) were used. Healthy volunteer samples ($n = 16$, M/F: 9:7, mean age: 36.9 ± 15.9 years) were from St George Hospital (University of New South Wales, Australia). Institutional ethics approval for blood collection was attained from each center and fully informed consent was taken prior to venepuncture for all subjects. All APS patients met the revised consensus classification criteria for APS [21]. Venous thrombotic events were diagnosed based on a combination of clinical assessment and appropriate imaging with either doppler ultrasonography or venography to document deep venous thrombosis (DVT) or isotope ventilation/perfusion scanning or computed tomographic (CT) imaging (± angiography) to confirm pulmonary embolism. Arterial events were diagnosed clinically along with a combination of one or more of the following - electrocardiographic evidence for myocardial ischemia or infarction, CT or MRI imaging of the brain to confirm infarction, doppler ultrasonography or angiography to confirm peripheral vascular disease or arterial embolus. All blood samples were stored at −80 °C until use.

2.10. Serial dilution of plasma for the detection of reduced FXI

50 μl of plasma was incubated with or without MPB (4 mM) at RT for 30 min in the dark with agitation, diluted 50 fold in 20 mM HEPES buffer, pH 7.4 (HBS) and incubated in the dark at RT for a further 10 min. Unbound MPB was then removed by acetone precipitation. The protein pellets were re-suspended in PBS-Tween (0.05%), the final dilutions were 62.5–8000 fold. Samples were then added to streptavidin 96-well plates 100 μl/well in duplicate and incubated at RT for 90 min. Before adding MPB labelled plasma samples, streptavidin plates were pre-washed three times with PBS-Tween (0.05%), and blocked with 2% BSA/PBS-Tween (0.1%). After washing three times with PBS-Tween (0.1%), a monoclonal goat anti-human FXI antibody was added (1:2000) and incubated at RT for 1 h. After washing three times with PBS/Tween (0.1%), ALP conjugated anti-goat IgG was added (1:1500) and incubated at RT for 1 h. After addition of chromogenic substrate samples were read at OD 405 nm.

2.11. Dose response of MPB -labelled FXI in normal plasma

MPB (0.2–8 mM) was added to 50 μl of normal plasma and incubated at RT for 30 min in the dark with agitation, and unbound MPB was removed by acetone precipitation. Detection of reduced FXI was done essentially as described above and the protein pellets were dissolved in PBS-Tween (0.05%) to a final dilution of 100 fold.

2.12. MPB-labelled FXI in normal and FXI deficient plasma

50 μl of normal or FXI deficient plasma was incubated with MPB (4 mM) at RT for 30 min in the dark with agitation. The procedure of removing free MPB and detection of reduced FXI was identical to that described above.

2.13. Detection of reduced FXI in FXI deficient plasma mixed with different percentage of normal plasma

FXI deficient plasma was mixed with different percentages (0–100%) of normal plasma. 4 mM of MPB was added to 50 μl of reconstituted plasma and incubated at RT for 30 min with agitation. The procedure of removing free MPB and detection of reduced FXI was identical to that described above.

2.14. Comparison of the relative level of reduced FXI between APS and normal subjects

To compare the amount of FXI in the reduced form between patients with APS and thrombosis and normal controls, we measured the relative amount of MPB-labelled FXI in these groups based on the ELISA assay described above. The pooled in-house standard derived from 10 healthy volunteers was used as an internal control and standard for each experiment. The same in-house pooled standard was used for each MPB labelling experiment to correct for any variation in MPB labelling. The degree of MPB-labelled FXI for each sample was expressed as a percentage of that observed with the pooled in-house standard, after correcting for the level of total FXI. Total FXI in plasma was detected using a commercial kit. Intra-plate co-efficient of variation (CV) was calculated by running 10 duplicates of the same sample on a single plate. Interplate-CV was calculated by taking 10 independent assays performed consecutively on separate days and calculating the CV based on the variation of the result obtained by dividing the OD of

the standard at 50 fold dilution over the OD of the standard at 100 fold dilution for each plate.

2.15. Statistical analysis

The GraphPad Prism program (version 4.0 for Windows, San Diego, CA) was used for the analysis of data. Data are expressed as mean ± SD. Differences between groups were compared by using 2-sided Student's *t* test. *p* values less than 0.05 were considered to be statistically significant.

3. Results

3.1. FXI is reduced by TRN or PDI/DTT

Human plasma derived FXI treated with or without reduced TRX-1/PDI and labeled by MPB was analyzed by Western blotting. Free thiols in proteins can be labeled with the biotinylated thiol-specific reagent MPB which can be detected by blotting with streptavidin–HRP. Purified FXI did not label with MPB, indicating that it does not contain accessible free thiols (lane 2, Fig. 1a). However, when FXI was treated with TRX-1/TRX-R/NADPH and incubated with MPB, 2 major and three minor labelled bands migrating between ~95–180 kDa and ~30–70 kDa respectively were detected with streptavidin-HRP, identifying that free thiols can be generated in purified FXI when treated with TRX-1 (lane 1, Fig. 1a). When probed with an anti-FXI antibody, untreated FXI showed a major band of 160 kDa (dimer) and a minor band of 80 kDa (monomer) (lane 2, Fig. 1b). In comparison, TRN-treated FXI showed two major bands at approximately 180 kDa and 100 kDa and several minor bands migrating between ~30–60 kDa (lane1, Fig. 1b). The immunoreactive band at 100 kDa of TRN-treated FXI may represent the monomer form of FXI as MPB labeling can significantly increase the molecular weight of proteins on SDS-PAGE as we have recently demonstrated for β2GPI [5,6]. The shift in molecular weight of MPB labeled FXI was also seen with the dimer as this migrated at a higher molecular weight than the unlabelled FXI (lane 2, Fig. 1b). This result indicates that TRX reduces disulfide bonds in the FXI homo-dimer.

3.2. TRX reduces the FXI Cys362–Cys482 and Cys118–Cys147 intra-chain disulfide bonds and the Cys321–Cys321 inter-chain disulfide bond

To identify the specific cysteines in FXI involved in the reaction with TRX-1, TRN-treated FXI incubated with or without MPB were resolved on SDS-PAGE and stained with silver (Fig. 1c). For these experiments, incubation of FXI with TRN was for 15 min. Three distinct bands (1–1, 1–2, 1–3, lane 1, Fig. 1c) were excised and subjected to mass spectrometry analysis. Of the three bands, MPB-labeled cysteines were found most significantly in the top band (1–1). The labeled cysteines include Cys362, Cys118 and Cys147 (Fig. 1d). Band 1–2 was found labeled at the same residues but to a significantly lower extent. No cysteines were demonstrated to be labeled in band 1–3. Band 1–3 is at the molecular mass for monomeric FXI (~80 kD).

This result indicates that TRX can reduce the Cys362–Cys482 and Cys118–Cys147 intra-chain and Cys321–Cys321 inter-chain disulfide bonds. Cys482 (of the Cys362–Cys482 disulfide bond) and Cys321 were not significantly labelled in our experiments, which is probably due to inaccessibility to MPB in the quaternary and tertiary structures, respectively. The differential labeling of Cys118 and Cys147 (Fig. 1d) is likely due to the same reason.

3.3. FXI reduced with TRX or PDI is activated more efficiently than the oxidised protein

TRN/PDI treatment increased the activation of FXI by both thrombin and FXIIa in the presence of various concentrations of DS (0.125–1 μg/ml, data not shown). The optimal concentration of DS was shown to be 0.25 μg/ml, for both TRX-1 and PDI treated FXI. FXI reduced by TRX-1 and activated by thrombin (Fig. 2a) or FXIIa (Fig. 2b) significantly ($p < 0.001$) enhanced generation of FXIa compared to HSA-treated FXI.

There is a significant increase in thrombin activation of FXI to FXIa when FXI is treated with TRX-1 or PDI, compared to HSA treatment in the presence of DS. There was a time dependent increase in activation seen at 15 min with generation of FXIa increasing linearly for both TRX-1 and PDI treated FXI ($p < 0.001$) (Fig. 2c and d).

Auto-activation of FXI occurs in the presence of DS alone without the presence of a serine protease like thrombin. To determine the effect of TRX-1 treatment of FXI on autoactivation we performed a time course experiment up to 60 min and at discreet time points the amount of FXIa generated was assessed. There was a lag time of approximately 30 min before FXIa generation was detected and there was a significantly increased rate of FXIa generation over the following 30 min by TRX-1-treated FXI compared to HSA-treated FXI ($p < 0.001$) (Fig. 2e).

TRN treatment of the serine proteases, thrombin, FXIIa and FXIa, had no effect on the catalytic constants for cleavage of peptide p-nitroanalide substrates (Table 1). This result supports our conclusion that TRN treatment of FXI results in increased conversion of FXI to FXIa, rather than direct effects on the catalytic activity of the activating enzymes.

3.4. Detection of reduced FXI in human plasma

In order to determine whether FXI is reduced naturally *in vivo*, an ELISA for detecting reduced FXI in plasma was developed. In this assay, fresh plasma is incubated with the thiol-specific biotin-linked alkylator MPB, unreacted MPB is removed by acetone precipitation, and the MPB-labelled proteins are collected on streptavidin-coated plates. Bound FXI is detected using a monoclonal anti-FXI antibody. The intraplate CV for this ELISA was 2.6% and interplate CV was 7.6%. A number of controls were used including non-MPB labelled human serum probed with anti-FXI (Fig. 3a) indicating that there was negligible signal (OD 405 nM < 0.1). An MPB dose–response was performed using normal human pooled plasma from 10 healthy volunteers. The signal plateaued at 4 mM MPB (Fig. 3b) and this concentration of alkylator was used in subsequent experiments. In order to be certain of the FXI specificity of this ELISA, FXI-deficient human plasma was used as a negative control. The ELISA detects a strong signal with MPB labelled normal human plasma (mean OD ± SD, 0.9 ± 0.06, $n = 3$) and negligible signal using MPB-labelled FXI deficient plasma (mean OD ± SD 0.12 ± 0.005, $n = 3$) (Fig. 3c). The specificity of MPB-labelling of FXI was further tested in mixing experiments with normal and FXI-deficient plasma. There was a dose-dependent increase in signal with increasing ratio of normal to deficient plasma (Fig. 3d).

Because EDTA has been reported to have an antioxidant effect [22], it is possible that this anticoagulant may influence the level of reduced FXI detected in plasma. Moreover, it should be noted that there are some differences in the clotting factors between plasma and serum. Therefore, it is essential to examine if the levels of circulating reduced FXI detected by this assay were different between the two preparations. To ascertain this, serum and plasma samples were prepared from the same person (healthy man, 30 years old) at the same venepuncture. The plasma was collected into VACUETTE®

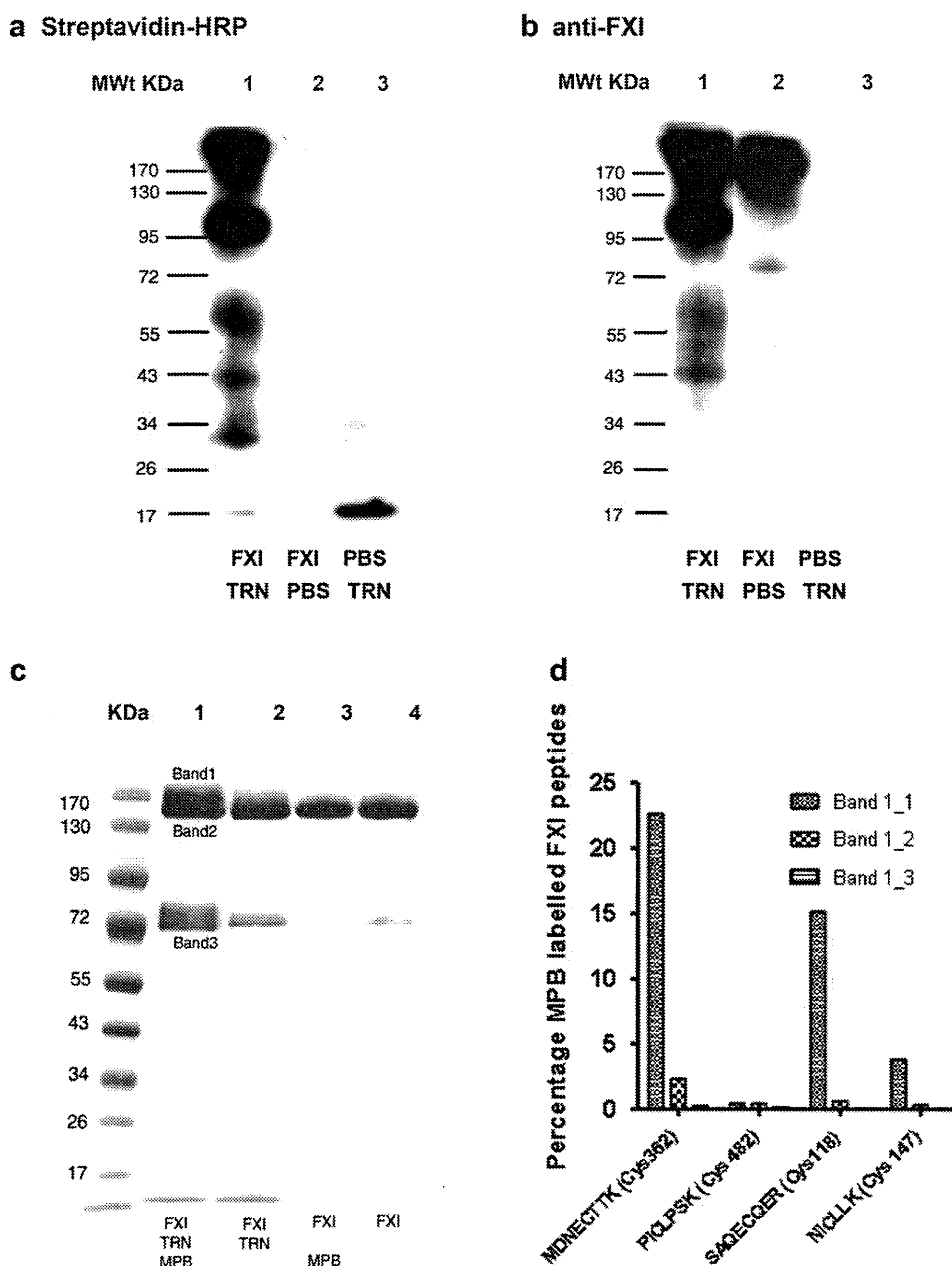

Fig. 1. Detection of free thiols in reduced FXI. FXI was incubated with TRX-1 activated with TRX-R/NADPH (TRN) as described in methods. Reduced FXI was labelled with the biotinylated thiol specific probe MPB and unreacted MPB was quenched with GSH. (a) Samples were resolved on SDS-PAGE under non-reducing conditions and transferred to PVDF membrane. Biotin labelled FXI was detected by probing with streptavidin–HRP. (b) The membrane was then stripped and incubated with goat anti-human FXI and rabbit anti-goat HRP to detect FXI. (c) Silver staining of FXI reduced with TRN and labelled with MPB. Controls used were FXI/TRN, FXI/MPB and FXI alone. The gel bands in lane 1 band 1, 2 and 3 were excised, digested and analysed by liquid chromatography tandem mass spectrometry. Mass spectral data were searched as indicated in supplementary methods. (d) Cys^{362} and Cys^{118} are the major labelled cysteines. The peptide sequences identified by mass spectrometry are shown for each of the cysteines labelled with MPB in FXI reduced with TRX-1.

EDTA K3 tube or coagulation tube containing 3.2% sodium citrate, respectively. Reduced FXI was estimated by ELISA as described above on blood samples in serial dilutions in parallel. The levels of reduced FXI in these samples were almost identical (data not shown).

3.5. There is increased proportion of reduced FXI in patients with APS

The ratio of reduced to total FXI levels was measured in patients with APS and thrombosis compared to normal controls, and patients with aPL autoantibodies but no thrombosis. Reduced FXI was compared and expressed as a percentage of that observed with a pooled standard (derived from 10 healthy volunteers age and sex matched with the APS group) after correction for total amount of FXI. Total FXI level was no different in APS or aPL positive and aPL negative patients without thrombosis, with a mean of ~20 μg/ml ($n = 10$ for APS, $n = 15$ for aPL negative and $n = 20$ for aPL positive without thrombosis) (Fig. 4a). The relative proportion of reduced FXI, expressed as a percentage of that observed with the in-house standard, is significantly increased in

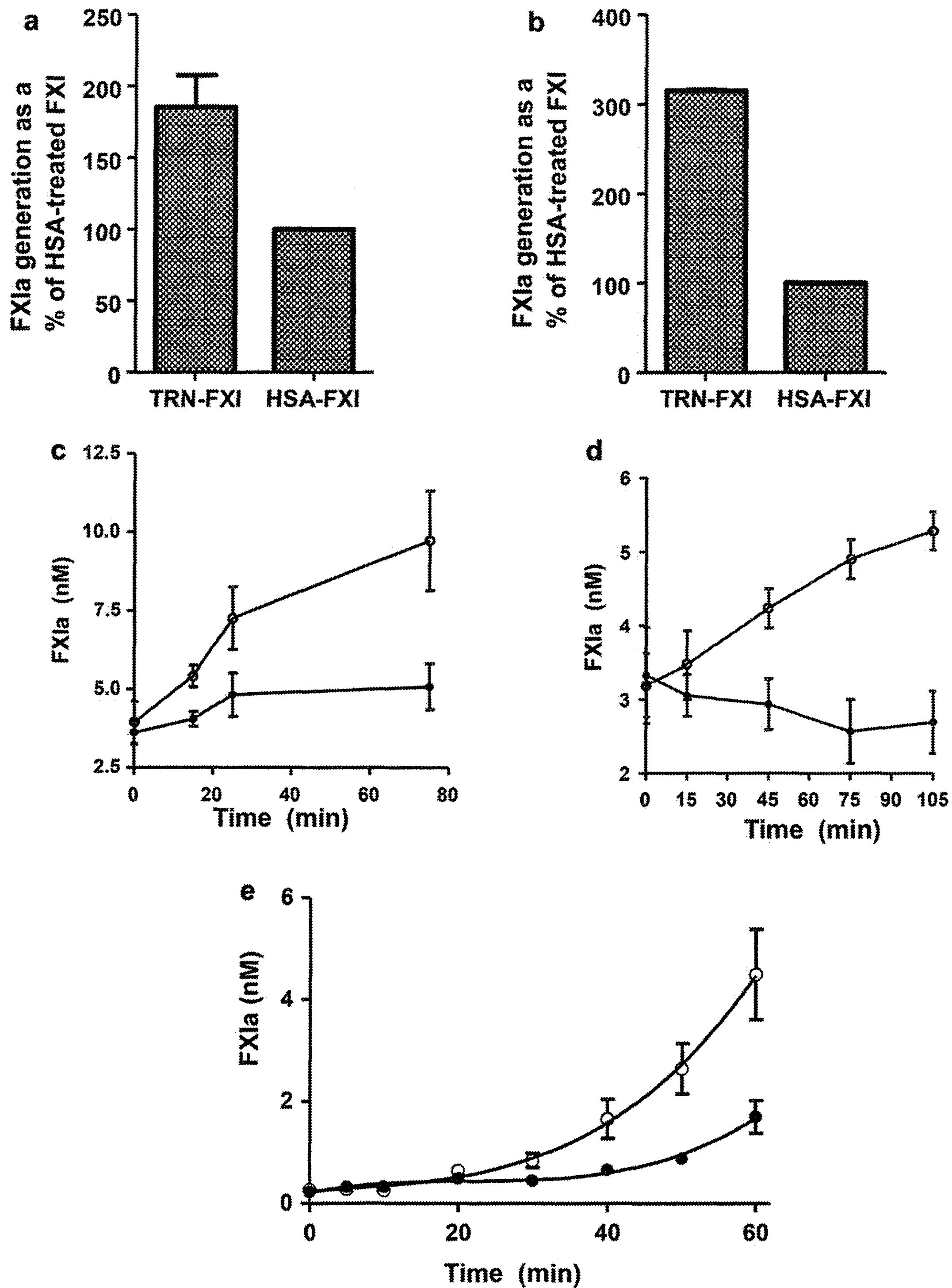

Fig. 2. Activation of TRX-1- or PDI-treated FXI with thrombin or FXIIa. TRN- or control HSA-treated FXI (60 nM) was activated by thrombin (2 nM, $n = 6$) (a) or FXIIa (2 nM, $n = 3$) (b) in the presence of DS (0.25 ug/ml) at 37 °C for 10 min. Reactions were stopped with hirudin (25 U/ml) and polybrene. The amount of FXIa generated was measured with the chromogenic substrate S2366. FXI was treated with TRN ($n = 4$) (c) or PDI/DTT ($n = 3$) (d) at 37 °C and aliquots (FXI: 60 nM) were withdrawn at indicated times and activated by thrombin (2 nM) in the presence of DS (0.25 ug/ml) for 10 min at 37 °C. Reactions were stopped with hirudin (25 U/ml) and polybrene. (e) Autoactivation of TRN- or control HSA-treated FXI (30 nM) in the presence of DS (0.25 ug/ml) at 37 °C. At indicated times, aliquots were withdrawn and mixed with polybrene (200 μg/ml) to neutralize DS. The generation of FXIa was measured with S2366. (○) = TRN+, (●) = TRN-.

APS patients who presented with vascular thrombosis as compared to healthy controls and aPL positive patients with no thrombosis ($p < 0.0001$ and $p < 0.001$ APS compared to aPL− and aPL+ with no thrombosis respectively) (Fig. 4b). Patients with APS and thrombosis, therefore, have increased levels of circulating reduced FXI.

It is important to note that the levels of reduced FXI in normal controls and pooled healthy subjects were almost identical although the average age of pooled healthy subjects was much lower than that of normal controls (53.7 ± 10.4 *vs* 36.9 ± 15.9 years), indicating the amount of reduced FXI in blood is stable and unaffected by age.

Table 1
Kinetic constants for hydrolysis of chromogenic substrates by HSA- or TRN-treated FXIa, FIIa and FXIIa.

Substrate	Enzyme	Km (uм)	Kcat (s^{-1})	Kcat/Km ($uм^{-1}$ s^{-1})
S2366	TRN-FXIa	470.7 ± 66.0	337.7 ± 17.2	0.72 ± 0.07
	HSA-FXIa	461.1 ± 36.6	343.6 ± 16.0	0.75 ± 0.03
S2238	TRN-FIIa	10.3 ± 3.2	221.6 ± 23.7	22.8 ± 5.9
	HSA-FIIa	8.9 ± 1.8	208.0 ± 1 6.1	24.0 ± 4.9
S2302	TRN-FXIIa	142.6 ± 8.5	19.6 ± 2.2	0.14 ± 0.01
	HSA-FXIIa	134.4 ± 4.0	20.6 ± 2.2	0.15 ± 0.02

4. Discussion

In the present study we have shown that FXI can be reduced by oxidoreductases TRX-1 and PDI, and that reduced FXI exists in normal human plasma. The reduced FXI is activated to FXIa more efficiently by thrombin, FXIIa and FXIa in the presence of DS. Notably, patients with APS thrombosis have higher plasma levels of reduced FXI than control groups.

FXI is composed of two identical polypeptide chains linked by an inter-chain disulfide bond (Cys321) within the A4 domain of each subunit. This homodimeric structure is unique among coagulation enzymes. FXIIa, α-thrombin, and FXIa (autoactivation) cleave FXI at a single peptide bond (Arg369-Ile370) and generate FXIa [19,20,23,24]. FXIa then activates FIX to FIXa by cleaving two scissile

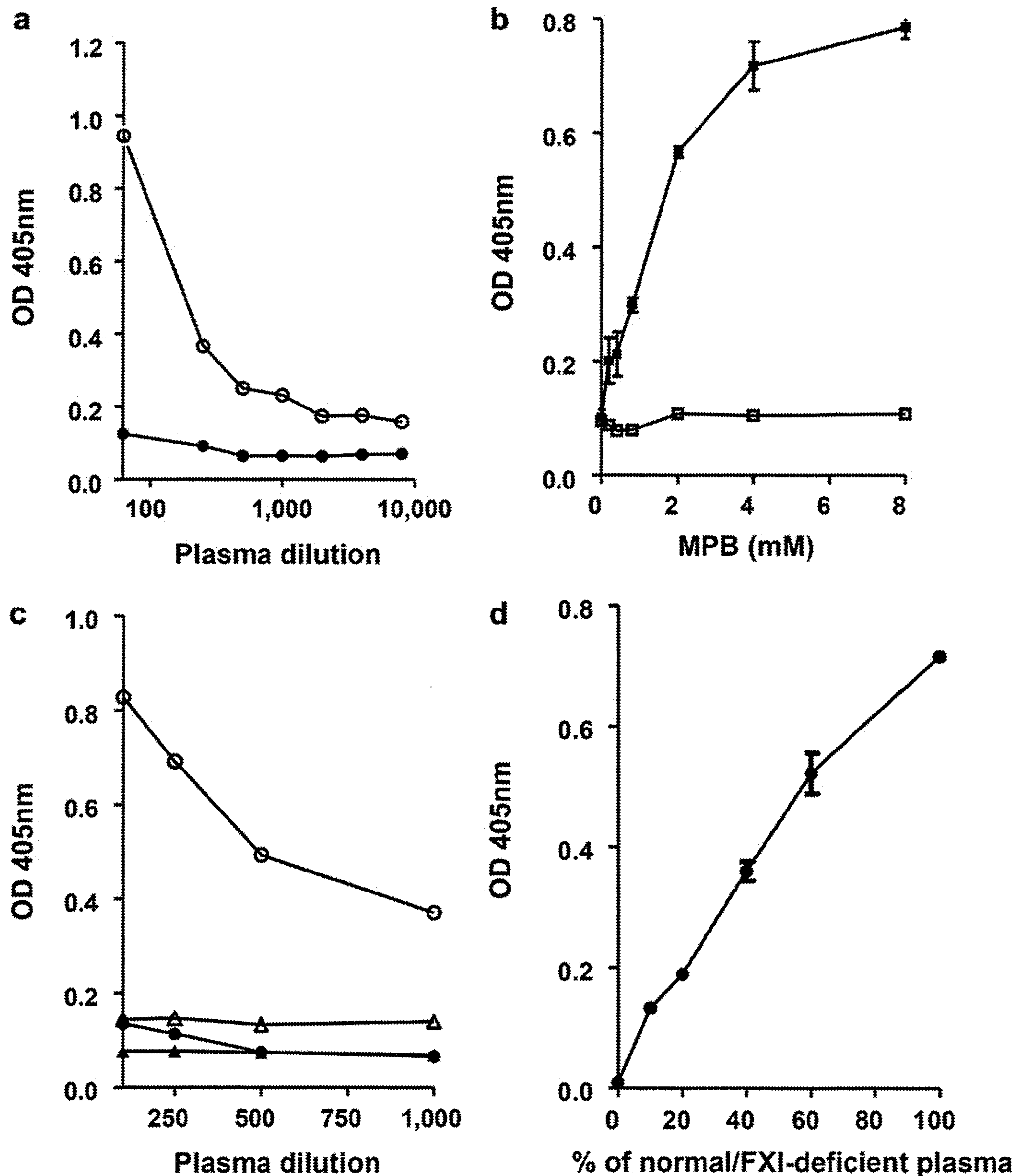

Fig. 3. Detection of naturally occurring free thiols in FXI in normal plasma (a) Serial dilution of plasma for detection of reduced FXI. MPB and non-MPB labelled plasma samples were acetone precipitated (to remove free MPB). The protein pellets were re-suspended in PBS-Tween (0.05%) to a final dilution from 62.5 to 8000 fold. Samples were coated on a streptavidin plate and probed with a monoclonal antibody to FXI. (○) = MPB+, (●) = MPB−. (b) Dose response of MPB-labelled FXI in normal plasma. A dose response of MPB (0.2–8 mм) was used to label plasma samples and the amount of reduced FXI was quantified with the use of the assay described above. (□) = MPB−, (■) = MPB+. (c) MPB-labelled FXI in normal and FXI deficient plasma. Normal (○, ●) or FXI deficient (△, ▲) plasma was incubated with or without MPB (4 mм) and the amount of reduced FXI was quantified using the assay described above. (○), (△) = MPB+, (●), (▲) = MPB−. (d) MPB labelled FXI in different % of normal plasma ($n = 2$). FXI deficient plasma was mixed with different percentage (0–100%) of normal plasma then incubated with MPB (4 mм) at 22 °C for 30 min. Detection of reduced FXI was assayed as described above.

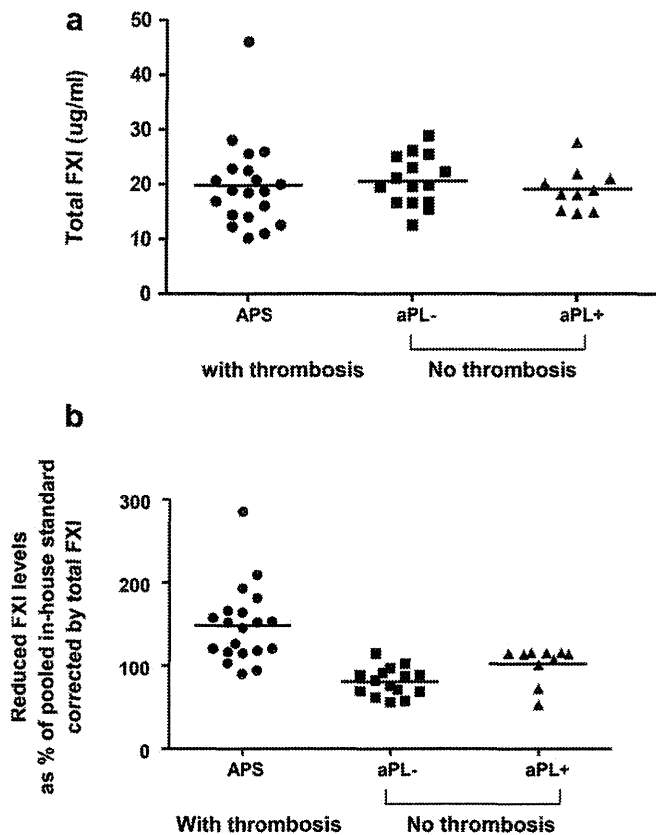

Fig. 4. Detection of total and reduced FXI in APS patients with thrombosis and aPL negative and aPL positive patients without thrombosis. (a) Total plasma FXI in the three groups was detected using a commercial kit. (b) Reduced FXI in plasma was detected by a streptavidin plate based ELISA (as described in methods) and expressed as a percentage of the pooled in-house standard corrected for total FXI.

bonds (Arg145–Ala146 and Arg180–Val181) [24]. Each of the four apple domains in the subunits of FXI contain six cysteine residues at highly conserved positions which form three disulfide bonds. Each apple domain consists of 7 β-strands that fold into a curved anti-parallel sheet cradling an α-helix [25]. Two disulfide bonds lock the helix onto the central β4 and β5 strands, whereas the other one links the N- and C-termini of the domain. In the present study, we show that the thiol oxidoreductases TRX-1 and PDI can reduce one or more disulfide bonds in FXI. The activation of reduced FXI by FXIIa, thrombin and FXIa, was enhanced dramatically compared to oxidized FXI. This is consistent with an influence on the conversion of FXI to FXIa, since TRX-1 treatment had no effect on the amidolytic activities of FXIIa, thrombin and FXIa.

To locate the specific disulfide bonds involved in thiol exchange reactions and elucidate the relevance to function, mass spectrometry was performed on FXI treated with TRX-1 and labeled with MPB. Three cysteines (Cys362, Cys118 and Cys147) could be labeled by MPB, Cys362 being the most heavily biotinylated cysteine residue. Cys362 participates in the formation of a disulfide bond with Cys482 and is six residues from the scissile Arg369-Ile370 peptide bond (Fig. 5). Cleavage of the bond appears to bring about a change in the conformation of FXI that renders it a more favorable substrate of thrombin, FXIIa and FXIa.

Antiphospholipid syndrome (APS) is a disorder characterized by thrombosis plus elevated and persistent titres of antiphospholipid antibodies (aPL) [21]. It is a common cause of acquired hypercoagulability [26]. In this study we found that the relative portion of reduced FXI is significantly higher in APS patients compared to healthy controls. We propose that the risk of pathological

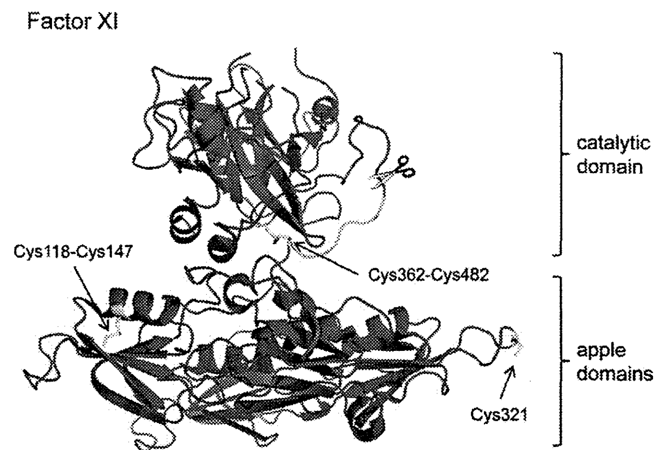

Fig. 5. Crystal structure of FXI and positions of the redox active cysteine residues. Ribbon structure (blue) of FXI showing the apple domains, catalytic domain and redox-active cysteine residues in stick representation (yellow). The Cys362–Cys482 disulfide bond cleaved by TRX is only six residues (in green) from the scissile Arg369–Ile370 peptide bond (in red). Reduction of the Cys362–Cys482 disulfide bond increases the efficiency of cleavage of the scissile peptide bond, presumably by triggering a favorable conformational change in the vicinity of the scissile bond. The structure is that of PDB ID 2F83 [32]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

thrombosis in APS may be attributed to elevated levels of the reduced form of FXI, as it can be more efficiently converted to FXIa than the oxidized form. A high FXI level is a risk factor for venous [3] and cerebrovascular thrombosis [4]. This pattern of thrombosis is seen in APS [2]. In animal models conversion of FXI to FXIa is critical for pathological thrombus propagation [27,28]. Attenuation of FXIa generation prevents vessel occlusion [27,28]. Murine experiments have shown that extracellular PDI is required for fibrin generation and platelet thrombus formation [29,30]. These published findings, in conjunction with our own, suggest that it is plausible to hypothesise that *in vivo* the amount of reduced FXI generated is increased as a result of PDI release, enhancing thrombus propagation by accelerating FXIa generation. Quantifying the amount of reduced FXI in patients positive for antiphospholipid antibodies may allow clinicians to predict those at increased risk for thrombosis, and allowing for implementation of preventative strategies. The need for incorporating tests of hyerprcoagulability to stratify thrombotic risk in patients who are positive for antiphospholipid antibodies has previously been suggested by Devreese [31].

4.1. Conclusions

The present study demonstrates that FXI can be reduced by oxidoreductase such as TRX-1. FXI in the reduced form demonstrates enhanced activation by thrombin, FXIIa and FXIa, leading to the generation of fully functional FXIa. APS patients with thrombus have significantly higher level of FXI in the reduced form when compared to healthy controls, which suggests that the redox state of FXI *in vivo* may be of pathogenic relevance, and this insight may provide a potential explanation for the high risk of thrombotic events in APS. Finally, from my group in Australia, it is an honor to contribute to this special issue dedicated to Pierre Youinou, as part of the Journal of Autoimmunity's series recognizing distinguished figures in autoimmunology; these have included Professors Harry Moutsopoulos, Ian Mackay, Chella David and Noel Rose [33–36].

Contributions

B.G., L.G., J.C.Q. and S.A.K. contributed to the experimental design. B.G., L.G., M.Q., D.M.Y., P.G.V., H.M.M., T.A., T.K., J.C.Q. collected data.

B.G., D.M.Y. and S.A.K. supervised the study. J.W.W., P. H. contributed new analytical tools and B.G., L.G., M.Q., J.W.W., P.G.V., H.M.M., T.A., T.K., P.H., J.C.Q. and S.A.K. contributed to writing the paper.

Disclosures/Conflict of interests

The authors declare no conflict of interest.

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All authors were involved in drafting the article, revising it critically for important intellectual content and all authors approved the final version to be published.

Dr. Krilis had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jaut.2012.05.005.

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REVIEW

Pathophysiology of thrombosis and pregnancy morbidity in the antiphospholipid syndrome

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ABSTRACT
In patients with the antiphospholipid syndrome (APS), the presence of a group of pathogenic autoantibodies called antiphospholipid antibodies causes arteriovenous thrombosis and pregnancy complications. To date, the pathogenicity of the antiphospholipid antibodies has been the focus of analysis. Recently, the antibodies were reported to be capable of direct cell activation, and research on the underlying mechanism is ongoing. The antiphospholipid antibodies bind to the membranes of vascular endothelial cells, monocytes and platelets, provoking tissue factor expression and platelet aggregation. This activation functions as intracellular signalling, independent of the cell type, to activate p38MAPK and the transcription factor NFκB. Currently, there are multiple candidates for the membrane receptors of the antiphospholipid antibodies that are being tested for potential in specific therapy. Recently, APS was reported to have significant comorbidity with complement activation, and it was proposed that this results in placental damage and cell activation and, therefore, could be the primary factor for the onset of pregnancy complications and thrombosis. The detailed mechanism of complement activation remains unknown; however, an inflammation-inducing substance called anaphylatoxin, which appears during the activation process of the classical complement pathway, is thought to be a key molecule. Complement activation occurs in tandem, regardless of the pathology of APS or the type of antiphospholipid antibody, and it is thought that this completely new understanding of the mechanism will contribute greatly to comprehension of the pathology of APS.

Keywords Antiphospholipid syndrome, cellular activation, complement activation.
Eur J Clin Invest 2012; 42 (10): 1126–1135

Introduction

Antiphospholipid syndrome (APS) is an autoimmune disorder defined by the persistent presence of antiphopholipid antibodies (aPL) in plasma of patients with vascular thrombosis and/or pregnancy morbidity. The clinical features and laboratory manifestations associated with aPL have considerably broadened since the first description of APS in 1983 [1] including thrombocytopenia, haemolytic anaemia, cardiac valve disease, pulmonary hypertension, nephropathy, skin ulcers, livedo reticularis, cognitive dysfunction and atherosclerosis [2].

An international consensus on classification criteria for APS was stated in Sapporo (Sapporo criteria) [3], and they were revised in 2006 in Sydney [4]. Definition of APS is made when at least one of the two clinical criteria (vascular thrombosis or pregnancy morbidity) occurs in a patient whose laboratory tests for aPL are positive (Table 1).

The relevant antibodies found in APS are directed against specific plasma proteins that possess an affinity for anionic phospholipids, such as β2 glycoprotein I (β2GPI) and prothrombin [5,6]. APL can be categorised into those antibodies detected by solid-phase enzyme-linked immunosorbent assays (ELISA) such as anticardiolipin antibodies, antiβ2GPI antibodies or those that prolong phospholipid-dependent coagulation time, called lupus anticoagulant.

There are two aspects of APS, vascular manifestations and pregnancy complications. Thrombus formation is the key event of vascular manifestations in APS, and many pathogenic mechanisms have been proposed to explain the thrombotic predisposition in this syndrome. However, obstetrical complications in patients with APS cannot be caused solely by thrombosis in the uteroplacental vasculature, and additional pathways have been raised to pregnancy problems in APS [7].

The mechanisms of thrombosis production in patients with APS are not completely clarified. However, the interaction between aPL and cells involved in the regulation of haemostasis is one of the mechanisms responsible of the thrombophilic state in APS. The aPL-cell interaction induces a perturbation in

Table 1 Revised classification criteria for the antiphospholipid syndrome [3]

Clinical Criteria
Vascular thrombosis
≥ 1 clinical episodes of arterial, venous or small vessel thrombosis, in any tissue or organ confirmed by objective validated criteria by imaging or histopathology in the absence of significant evidence of inflammation in the vessel wall
Pregnancy morbidity
≥ 1 unexplained deaths of a morphologically normal foetus at or beyond the 10th week of gestation, or
≥ 1 premature births of a morphologically normal neonate before the 34th week of gestation owing to eclampsia, severe pre-eclampsia or placental insufficiency, or
≥ 3 unexplained consecutive spontaneous abortions before the 10th week of gestation (maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded)
Laboratory Criteria
Lupus anticoagulant present in plasma, on ≥ 2 occasions at least 12 weeks apart, detected according to the guidelines of the International Society on Thrombosis and Haemostasis
IgG and/or IgM anticardiolipin antibodies present in medium or high titre in serum or plasma, on ≥ 2 occasions at least 12 weeks apart, measured by a standardised ELISA
IgG and/or IgM antiβ2 glycoprotein I antibodies present in titre >99th percentile, in serum or plasma, on ≥ 2 occasions at least 12 weeks apart, measured by a standardised ELISA

Antiphospholipid syndrome is present if at least one of the clinical criteria and one of the laboratory criteria are met.
ELISA, enzyme-linked immunosorbent assay.

the cells that results in a pro-thrombotic/pro-inflammatory response and subsequently thrombosis.

Complement activation, one of the mechanisms related to obstetric complications in APS has also been involved in the production of thrombosis in patients with aPL. In this manuscript we discuss the aPL-cell interaction and the role of complement in the aPL-associated complications as the major pathogenic mechanism (Table 2).

Antiphospholipid antibody-associated thrombosis

The association between aPL and thrombotic events is well established. Evidence from animal models of APS indicates that aPL may play a causal role in the vascular abnormalities in both the venous and arterial territories [8,9]. In an animal model of photochemically induced arterial thrombosis, monoclonal antibodies raised against human β2GPI promoted thrombus formation [8]. Ramesh *et al.* [9] demonstrated that aPL inhibit the activation of nitric oxide and that the resulting decline in nitric oxide production underlies the promotion of leucocyte–endothelial cell (EC) adhesion and arterial thrombosis in mice. Injection of aPL in mice increased thrombus formation, carotid artery tissue factor (TF) activity, as well as peritoneal macrophage TF activity and expression [10]. Furthermore, enhanced thrombus formation was observed in femoral vein of mice treated with aPL [11]. Vega-Ostertarg *et al.* [12] found that mice injected with aPL have an enlargement in the thrombus size in the postcapillary venular endothelium in the cremaster muscle. Rapid endothelial deposition of fibrinogen and intravascular platelet–leucocyte aggregates were detected by intravascular microscopy on the mesenteric vessels of rats receiving an intraperitoneal injection of bacterial lipopolysaccharide followed by infusion of immunoglobulin G (IgG) purified from patients with APS [13].

Despite the persistent presence of aPL in circulation, thrombotic events in patients with aPL only occur occasionally, suggesting that the presence of aPL is necessary but not sufficient for clot formation *in vivo*. The 'two-hit hypothesis' has been proposed in which aPL (first hit) can only exert their prothrombotic influence in the presence of another thrombophilic condition (second hit). This 'two-hit hypothesis' was shown in an animal model of APS in which the injection of aPL in rats only resulted in increased thrombus formation when rats were pretreated with lipopolissacharide, but not when were injected with buffer [13].

Antiphospholipid antibodies and cell interactions

The major antigen structures recognised by aPL in patients with APS are phospholipid–binding proteins, β2GPI and prothrombin, expressed on the membranes of different cell types. The antibody forms a complex with the corresponding antigen,

Table 2 Antiphopholipid antibodies (aPL)-mediated pathogenic mechanisms

aPL-mediated thrombosis
Interference with the components of the coagulation cascade
Protein C pathway
Protein Z pathway
Contact activation pathway
β2GPI-thrombin interaction
Impairment of fibrinolysis
Cell interaction
Induction of proinflammatory phenotype on endothelial cells
Induction of procoagulant activity on endothelial cells and monocytes
Release of membrane-bound microparticles
Pro-coagulant effects on platelets
Disruption of the annexin V shield
Complement activation
aPL-mediated foetal loss
Intraplacental thrombosis
Inflammation
Inhibition of syncitium-trophobalst differentiation
Disruption of the annexin V shield
Complement activation

β2GPI, β2 glycoprotein I.

leading to the cell perturbation, the activation of cell signalling pathways, the transcription of procoagulant substances, adhesion molecules and subsequently thrombus formation.

Studies on the pathogenicity of aPL have been carried out mainly on the corresponding target molecules especially on the function of β2GPI and their modifications by aPL. However, to evaluate whether antiβ2GPI antibodies can block the function of β2GPI is difficult as true physiological role of β2GPI in coagulation cascade is not elucidated. Individual with complete β2GPI deficiency does not have any particular phenotype [14]. Thus, the recent trend is to favour the hypothesis that the function of aPL on prothrombotic cells, via β2GPI, is more important than the function of β2GPI.

Membranes of activated platelets with negatively charged phospholipids are an important source of catalytic surface for blood coagulation. Activated factor X and thrombin are generated on activated platelets, and procoagulant microparticles shed by platelet activation. Platelets are prone to agglutinate and aggregate after exposed to aPL [15], and circulating activated platelets are found in patients with APS [16]. β2GPI binds to membranes of activated platelets and inhibit the generation of activated factor X. Antiβ2GPI antibodies interfere with this inhibition [17]. Thus, activated platelets may be a predominant immune target of antiβ2GPI antibodies and direct action of aPL in platelets contribute to APS-related thrombosis.

The endothelium is a predominant target of aPL. Pathogenic aPL binding to β2GPI cause the up-regulation of adhesion molecules [18], TF [19] and endothelin-1 [20] causing a pro-inflammatory and prothrombotic EC phenotype. Prothrombin also binds to ECs, and this binding is enhanced by a human monoclonal IgG antiprothrombin antibody, IS6. IS6 up-regulates expression of TF and E-selectin on ECs [21].

Antiphospholipid antibodies exert also effect in the stimulation of the release of microparticles from ECs [22]. Microparticle production is a hallmark of cell activation, but the role of microparticle in the pathophysiology of thrombosis has not been elucidated. Antiphospholipid antibodies bind to the negatively charged membrane of monocytes and induce TF up-regulation [23,24]. Monocytes are the source of most majority of circulating TF-bearing microparticles [25] and TF up-regulation is a major feature of monocyte activation in the APS [26].

Cell receptors for antiphosphospholipid antibody interactions

The cell activation mediated by aPL might require an interaction between phospholipid-binding plasma protein and a specific cell receptor(s). A number of potential receptors for the binding of β2GPI to cellular membranes have been identified including annexin A2, apolipoprotein E receptor 2 (ApoER2′), low-density-lipoprotein receptor (LDL-R) -related protein, megalin, Toll-like receptor (TLR) 2, TLR 4, the very-LDL-R and P-selectin glycoprotein (GP) ligand-1. β2GPI also directly binds to the platelet adhesive receptor GPIbα and to the platelet factor 4 (PF4) [27–32]. Most of these receptors are expressed on various cell types and whether those different receptors are involved in the pathophysiology of thrombosis is still matter of debate.

Annexin A2 is a receptor for tissue-type plasminogen and its ligand plasminogen. Annexin A2 is a membrane-bound protein found on the surface of ECs and monocytes, and on the brush-border membrane of placental syncytiotrophoblasts [33]. Annexin A2 interacts with the β2GPI-antiβ2GPI antibody complex on the ECs and monocyte surfaces, mediating cell activation [27,28]. The involvement of annexin A2 in aPL-mediated pathogenic effects has been reported *in vitro* and *in vivo* models [34]. However, it is unlikely that annexin A2 *per se* is actually involved in cellular activation because it lacks transmembrane domain. The activation of signalling responses requires the presence of another transmembrane adaptor protein(s) that associates with annexin A2 on the ECs surface [29]. TLR-4 was

identified as a potential putative adaptor protein for annexin A2 [28].

Several groups reported that TLR-2 and TLR-4 are involved in aPL-mediated cell activation [30,35,36]. TLR-4 signalling was shown in ECs after the incubation with aPL [29], but a direct interaction between TLR4 and β2GPI remains to be confirmed. Binding of β2GPI to TLR2 on endothelial surface has been reported [37].

Megalin/gp33 is an endocytic receptor that internalises multiple ligands including apolipoprotein E and B100. Megalin was shown to behave as a receptor of β2GPI and β2GPI-phospholipid complex [38]. Pennings *et al.* [39] demonstrated that dimeric β2GPI can interact with LDL-R family members, including megalin.

Apolipoprotein E receptor 2 is a member of the LDL-R family expressed in many cell types. Studies on platelets suggested ApoER2′ as a receptor of β2GPI [40]. The blockage of the platelet ApoER2′ using a receptor-associated protein abrogated the increased adhesion of platelets to collagen induced by β2GPI-anti-β2GPI antibody complex [41]. Using a recombinant soluble form of LDL-binding domain 1 of ApoER2′, it was shown that the interaction between β2GPI and ApoER2′ mediated the aPL action in endothelium [9]. The importance of ApoER2′ in the induction of prothrombotic state mediated by aPL was confirmed *in vivo* in a murine model of thrombosis and using ApoER2′ deficient mice [42]. Injection of aPL caused a significant increase in thrombus formation, vascular TF activity and monocyte activation in the murine model of thrombosis, which were significantly reduced in the ApoER2′ deficient mice. Those data support the role of ApoER2′in thrombus formation in APS; however, the role of other potential receptors cannot be excluded as demonstrated by the partial protection from thrombogenic effects of aPL in ApoER2′-deficient mice.

β2 glycoprotein I directly binds to GPIbα subunit of the platelet adhesion receptor GPIb/IX/V *in vitro* [35,36]. The platelet GPIbα subunit has the von Willebrand factor as the most important ligand, but also serves to localise factor XI and thrombin on the platelet surface. Binding of β2GPI to GPIbα enables antiβ2GPI antibodies, directed against domain I, to activate platelets, resulting in thromboxane production and also to the activation of the phosphoinositol-3 kinase (PI3-kinase)/Akt pathway [31] contributing to the platelet adhesion and aggregation.

The involvement of Fcγ receptor on cellular activation has been investigated *in vivo* [8] and *in vitro* studies on platelets [26], monocytes [26] and ECs [28]. Results suggest that this receptor is not strictly necessary for cellular activation.

The direct binding of β2GPI to PF4 derived from platelet granules has been reported [43]. PF4 is a member of the C–X–C chemokine family secreted by activated platelets and has ability to bind to the platelets surfaces. PF4 contributes to the natural dimerisation of β2GPI, leading to the stabilisation of β2GPI binding onto the phospholipid cell surfaces which facilitates the antibody recognition. The β2GPI-PF4 complex is strongly recognised by serum of patients with APS [43]. Moreover, platelets may be activated by β2GPI-antiβ2GPI antibody-PF4 or β2GPI-PF4 complexes. Almost every cell type can be a source of PF4 especially under some stimulation. Both, β2GPI and PF4 are abundant in plasma; thus, the preformed β2GPI-PF4 complexes may prime several pro-coagulants cells culminating in coagulation.

Those potential receptors proposed to be involved in the aPL-mediated cell activation have significantly increased in the last years, and additional studies are needed to clarify their biological and pathological roles.

Signalling pathways of cell activation

The signal transduction mechanisms involved in aPL-mediated cell activation have been the centre of interest for many researchers. How pathogenic aPL recognition of phospholipid-binding proteins on the cell surface elicits a transmembrane signal to modify intracellular events is not completely understood.

The adapter molecule myeloid differentiation protein (MyD)88-dependent signalling pathway and the nuclear factor kappa B (NFkB) have been involved in the ECs activation by aPL [44,45]. Incubation of ECs with antiβ2GPI antibodies resulted in a redistribution of NFkB from the cytoplasm to the nucleus, and this effect was accompanied by an increased expression of TF and leucocyte adhesion molecules [46]. The p38 mitogen-activated protein kinase (MAPK) pathway is an important component of intracellular signalling cascades that initiate various inflammatory responses. It is recognised that the p38 MAPK pathway has a crucial role in mediating the effect of aPL in different cell types [24,47,48]. Activation of p38 MAPK increases activities of cytokines such as tumour necrosis factor (TNF) alpha, IL-1β and macrophage inflammatory cytokine 3β [24,36]. Monocytes stimulated by monoclonal antiβ2GPI antibodies from patients with APS induce phosphorylation of p38 MAPK, a locational shift of NFkB into the nucleus and up-regulation of TF expression. Such activation was not seen in the absence of β2GPI, indicating that the disturbance of monocyte by anti-β2GPI antibodies is started by interaction between the cell and the autoantibody-bound β2GPI [24,44]. The implication of p38 MAPK in cell activation has been also demonstrated in platelets [47] and ECs [48]. Pretreatment of platelets with p38 MAPK-specific inhibitor, SB203580, completely abrogated aPL-mediated platelet aggregation. The induction of TF expression was also reported through the simultaneous activation of NFkB via the MAPK pathway and of the MEK-1/ERK pathway, but an inhibitor of the MEK-1/ERK pathway could not suppress the TF

expression, implying the main role of p38 MAPK in those reactions [49].

Purified IgG from APS patients with venous thrombosis, without pregnancy morbidity, caused phosphorylation of NFkB and p38MPK and up-regulation of TF in monocytes. These effects were not seen with IgG fractions from patients with obstetric APS alone, suggesting that aPL from patients with different clinical aspects of APS may trigger different signalling responses [44]. Figure 1 shows the procoagulant cell activation as one of the pathogenic mechanisms of thrombosis mediated by aPL.

Recently, two major findings in the antigenic structures recognised by aPL have been reported: first, the structural changes in β2GPI. β2GPI can exist in two different conformations, plasma β2GPI circulates in a circular (closed) conformation, whereas after interaction with antiβ2GPI antibodies undergoes a major conformational change into a fishhook-like (open) structure [50]. Second, the finding that β2GPI can be reduced by thioredoxin 1 (TRX-1). β2GPI treated with TRX-1 generate free thiols within β2GPI, a process that may affect the function of β2GPI, and may have a regulatory role in platelet adhesion [51]. Those novel biochemical findings into the structural changes that can occur within β2GPI and the consequences of these changes for the function of β2GPI might be relevant to our better understanding of the APS, but further studies are necessary to clarify their roles in the pathogenesis of APS.

Complement activation in APS

Several genes involved in SLE susceptibility have recently been identified and confirmed. Of those, particularly *IRF5* [52] and *STAT4* [53] have been confirmed in several studies to be clearly associated with primary APS. In a study by our institution on gene polymorphisms of *STAT4* in Japanese primary patients with APS, the polymorphism rs7574865G/T was related to APS and *STAT4* was considered a disease-susceptibility gene for both SLE and APS regardless of race [54]. Of the similarities between SLE and APS, the focus in recent years has turned to complement activation as a common point that has been observed in the mechanism of pathology of both diseases [55].

Hypocomplementemia caused by complement activation is commonly observed in SLE and is thought to be well correlated with disease activity. Many proposed hypotheses have explained the involvement of the hypocomplementemiathis in the disease onset mechanism in SLE. Understanding the mechanism of hypocomplementemia and complement activation in

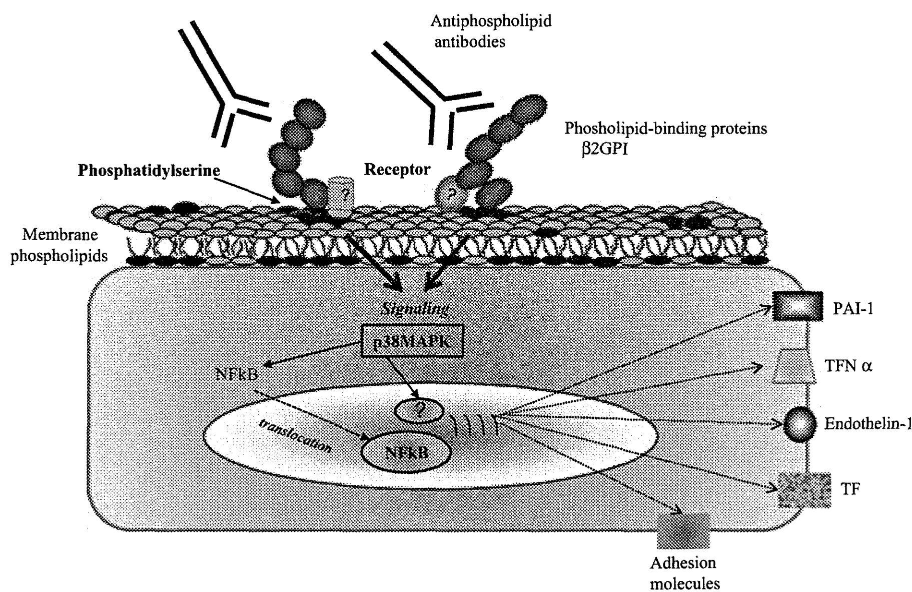

Figure 1 Pathogenic mechanisms of cell activation mediated by antiphosphopholipid antibodies. Antiphospholipid antibodies interact with monocytes or endothelial cells through binding to phospholipid-binding protein (β2GPI or prothrombin) on cell surface. This interaction might require a specific cell receptor (s) and results in p38MAPK phosphorylation, nuclear translocation of NFkB and up-regulation of procoagulant substances and adhesion molecules, and subsequently thrombus formation. p38 MAPK, p38 mitogen-activated protein kinase; NFkB, nuclear factor kappa B; β2GPI, β2 glycoprotein I; PAI-1, plasminogen activator inhibitor-1; TNFα, tumour necrosis factor alpha; TF, tissue factor.

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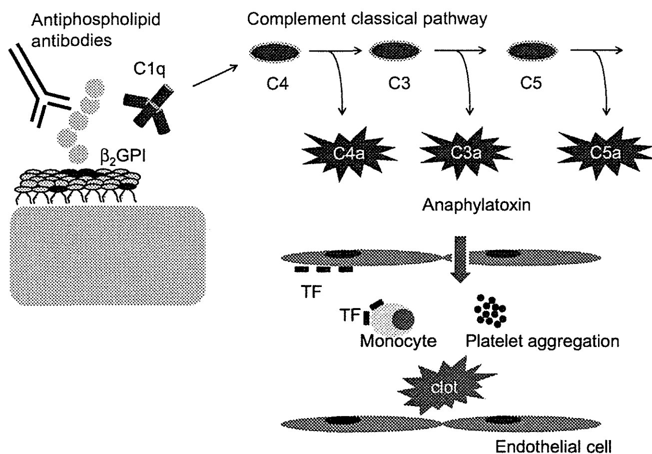

Figure 2 Pathogenic mechanisms of complement activation in antiphospholipid syndrome. Complement classical pathway which is initiated by C1q protein is significantly activated in the serum of patients with antiphospholipid syndrome. Activation of the pathway proceeds as cascade reaction producing anaphylatoxins, the fragments of the complement proteins that amplify the activation of monocytes, platelets or endothelial cells. Activation of these cells and molecules induces expression of TF or adhesion molecules and platelet aggregation. β2GPI, β2 glycoprotein I; TF, tissue factor.

APS is important because it has the potential to be helpful not only in understanding the pathology of APS but also in clarifying the involvement of the mode of complement activation in SLE as well.

Complement system

'Complement' is a general term for over 20 serum proteins that function in innate immunity. They would attach to the membrane of organisms and would be activated sequentially in cascade process. They are activated by pattern recognition receptors that have evolved to recognise specific molecular patterns. The complement system has three known activation pathways according to the differences of activation elements: the classical pathway activated by an antigen–antibody complex; the lectin pathway activated by lectin, recognising glycans on microorganisms; and the alternative pathway, in which C3 is bound without recognition molecules. These three pathways culminate in activation of C3, the central step of complement activation. Activation of C3 leads to the production of pro-inflammatory fragment C3a which is called anaphylatoxin and sequentially activates C5. Activation of C5 also produces anaphylatoxin C5a which has especially strong inflammation-inducing effect. C4, a classical pathway protein, also produces C4a on its activation which is another member of anaphylatoxin but has little effect on inducing inflammation. C5b, an another fragment of activated C5, forms a complex with C6, C7, C8 and C9 which is called membrane attack protein (MAC). MAC produces a trans-membrane channel on the membrane of microorganism that disrupts the phospholipid bilayer, leading to cell lysis and death.

Complement activation in APS pregnancy morbidity

Complement activation was first thought to be involved in the pathophysiology of pregnancy complication in APS. Initially, it was believed that thrombosis in placentas was an important mechanism of pregnancy complication in APS. Obstructed blood flow in placenta caused by thrombosis may impair foetal growth because of exchange failure of blood between mother and foetus [56]. Placental thrombosis has been reported, and *in vitro* studies have confirmed that aPL have the ability to disrupt the annexin A5 anticoagulant shield on trophoblast and EC monolayers [57]. However, other reports were unable to confirm the occurrence of multiple thrombosis in the placentas of patients with APS; in fact, placental thrombosis imaging was either virtually absent or only mild in the analysis of many cases [58].

A recent study pinpointed the trophoblastic basement membrane as a target of aPL, where the aPL were thought to complicate pregnancy by directly inducing localised inflammation. Multiple peritoneal injections of human IgG with aPL activity to pregnant naïve mice enabled embryo implantation and induced considerable placental damage that resulted in foetal resorption and growth retardation. Deposits of human-derived IgG and complement were observed in a pathological study of the placentas of these mouse models, accompanied with local TNF secretion and a temporary but a clear increase in serum TNF levels [59–61].

Obstetrical problems were markedly suppressed in these models when complement was deficient or when antibodies against the complement were administered, such that complement activation was thought to be an important mechanism in pregnancy complications by aPL [62].

The anaphylatoxin C5a is especially important and causes placental damage, having a strong inflammation-inducing effect that causes localised placental inflammation and promotes TF expression in neutrophils infiltrated to the placenta [63,64]. In addition, anti-complement activation is involved in the effect of heparin preventing aPL-related pregnancy complications [65]. However, considering the fact that the mouse models were given large doses (10 mg each) of IgG fractions containing aPL in human serum, it is unclear whether they adequately reflect the behaviour of aPL *in vivo* in human.

The involvement of complement activation in pregnancy morbidities in patients with APS has been vigorously studied, with a series of reports suggesting a clear involvement

in mouse models. In one retrospective study, complement deposition was found in the placental tissues of women positive for aPL [66]. There are some reports discussing the dysfunction of complement-regulating factor. Complement-regulating factor mutations are related to preeclampsia in patients with SLE or APS [67]. Decreased expression of decay-accelerating factor (CD55, a complement regulatory factor) in the endometrium has been confirmed in aPL-positive pregnancies (by endometrial biopsy) [68]. However, there remains a lack of conspicuous evidence that complement activation is directly related to the APS pregnancy morbidity outcome. Preliminary data from recent reports indicate that the histology of placental specimens from patients with APS shows evidence of complement activation compared with control placental specimens; however, complement deposition can be detected both in abortive specimens and in placentas at term without a clear relationship with either pregnancy outcome or therapy [45].

Although large prospective analyses are needed to demonstrate definite conclusions about the involvement of complement in APS-related pregnancy morbidity, the potential role of complement in aPL-mediated clinical manifestations should not be neglected. In addition to causing acute local inflammation, complement components are able to modulate the functions of procoagulant cells (monocyte, ECs) and decidual or trophoblast cells [69].

Complement activation in APS thrombosis

According to the positive results showing the relationship between complement activation and pregnancy morbidity in the APS murine model, an intensive study was carried out that investigated the relationship between thrombosis and complement activation in this model.

Mice deficient in C3 ($C3^{-/-}$) and C5 ($C5^{-/-}$) were used to investigate the role of complement activation in APS thrombosis. Each was administered aPL–IgG or control IgG, and thrombosis was induced via standardised pinch injury to the femoral vein [70]. The sizes of the thrombi in $C3^{-/-}$ and $C5^{-/-}$ mice were significantly reduced compared to those of wild-type mice. Additionally, mice treated with monoclonal anti-C5 antibody developed smaller thrombi compared with mice that did not receive the monoclonal antibody [71,72]. Complement activation using C5a production observed in the aPL-administered mice was found to induce TF expression on neutrophils, resulting in modified prothrombin time [71]. These phenomena suggest a possible mechanism by which aPL activation of complement pathway can initiate coagulation. Finally, antiβ2GP1 antibodies were found to initiate thrombus formation, with decreased thrombotic occlusions in C6-deficient rats and in mice treated with anti-C5 antibody [21].

A recent report investigated the significance of complement activation in patients with primary APS [72]. From the analysis of serum complement levels (C3, C4, CH50) and anaphylatoxins (C3a, C4a, C5a) in patients with primary APS, non-SLE connective tissue disease and healthy subjects, it revealed that complement levels were significantly lower in patients with primary APS compared with those with the other groups. Most patients with primary APS showed elevated serum levels of C3a and C4a related to hypocomplementemia. Among the patients with primary APS, no correlation was found between any particular clinical manifestation and hypocomplementemia. Hypocomplementemia is frequently found in patients with primary APS, reflecting complement activation and consumption rather than deficiency as suggested by the correlation between high serum C3a concentrations and low serum C3 levels. This conclusion strengthens the recognition of crosstalk between complement activation and prothrombotic status in APS (Fig. 2).

Crosstalk between complement and coagulation pathways

There is lesser evidence of APS thrombosis and complement activation than pregnancy complications, but various aspects of involvement have become known for complement activation and thrombosis. Complement activation is increasingly being recognised as a major contributor of vascular inflammation [73]. Complement deposition has been frequently observed in atherosclerotic lesions [74], and accumulating evidence suggests that complement plays a significant role in ischaemia/reperfusion injury [75]. C3a and C5a enhance leucocyte recruitment and support the host inflammatory response [76]. C5a level elevations have been associated with increased cardiovascular risk in patients with advanced atherosclerosis [77].

Recently, the complement pathway has also been identified as having an effect on the coagulation pathway itself. Both have highly substrate-specific reactions that proceed with the cascading activation of many different serine proteases, but some reactions also cross from one cascade to the other. For example, activated factor XII (FXIIa), an initiator of the intrinsic coagulation pathway, degrades and activates C1, an initiator of the classical complement pathway [78], while thrombin directly degrades C5 in the absence of C3 to produce the anaphylatoxin C5a [79]. In addition, C5a increases the expression of TF [80], and the membrane attack complex degrades prothrombin to thrombin [81]. Thus, the complement and coagulation pathways have a close relationship. This mechanism is very effective at the site of trauma by causing anaphylatoxin production at place of haemostasis; complement activation induces inflammation at the trauma site and effectively prevent microorganism infiltration. It is possible that a complex combination occurs

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in the involvement of complement activation in APS thrombosis, such as with vascular injury, direct activation of the coagulation pathway or cellular activation.

Conclusion

Ongoing research focused on cell receptors and intracellular signalling pathways involved in the cell activation mediated by aPL substantially advance the understanding of the thrombotic mechanism in APS. Further studies are needed to clarify the biological role of the numerous potential receptors proposed for aPL–cell interaction.

Complement activation seems to be an essential factor for disease manifestation in pregnancy morbidity in patients with aPL from the results of the experiments in *in vivo* models. Accumulating evidences are offering promising prospects on the involvement of complement activation in thrombosis related to aPL. Although the definite conclusion that complement activation is a part of the process of disease manifestation can only be induced from the result of large prospective studies, there is no doubt that the clarification of the mechanism of complement activation in APS would be a key to a better understanding of pathogenesis of APS.

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Novel Assays of Thrombogenic Pathogenicity in the Antiphospholipid Syndrome Based on the Detection of Molecular Oxidative Modification of the Major Autoantigen β_2-Glycoprotein I

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Objective. **Beta-2-glycoprotein I (β_2GPI) constitutes the major autoantigen in the antiphospholipid syndrome (APS), a common acquired cause of arterial and venous thrombosis. We recently described the novel observation that β_2GPI may exist in healthy individuals in a free thiol (biochemically reduced) form. The present study was undertaken to quantify the levels of total, reduced, and posttranslationally modified oxidized β_2GPI in APS patients compared to various control groups.**

Methods. **In a retrospective multicenter analysis, the proportion of β_2GPI with free thiols in serum from healthy volunteers was quantified. Assays for measurement of reduced as well as total circulating β_2GPI were developed and tested in the following groups: APS (with thrombosis) (n = 139), autoimmune disease with or without persistent antiphospholipid antibodies (aPL) but without APS (n = 188), vascular thrombosis without APS or aPL (n = 38), and healthy volunteers (n = 91).**

Results. **Total β_2GPI was significantly elevated in patients with APS (median 216.2 μg/ml [interquartile range 173.3–263.8]) as compared to healthy subjects (median 178.4 μg/ml [interquartile range 149.4–227.5] [$P < 0.0002$]) or control patients with autoimmune disease or vascular thrombosis (both $P < 0.0001$). The proportion of total β_2GPI in an oxidized form (i.e., lacking free thiols) was significantly greater in the APS group than in each of the 3 control groups (all $P < 0.0001$).**

Conclusion. **This large retrospective multicenter**

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