Detection of Anti-Domain I *β*-2 Glycoprotein I Antibodies as New Potential Target in Antiphospholipid Syndrome Diagnosis

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Abstract

Anti-cardiolipin antibodies (ACL) and anti-β-2-glycoprotein I antibodies (anti-2GPI) represent two out of three laboratory criteria for detection of antiphospholipid syndrome (APS). The domain I (DI) in anti-β-2-glycoprotein I is a new target for better identification of antibodies and may be associated with thrombotic risk in antiphospholipid syndrome.

Anti-β2GPI antibodies specifically reacting with DI have a particular clinical importance being more commonly detected among patients with APS and other autoimmune diseases. This observation implies that compared with antibodies targeting the whole molecule, anti-DI antibodies have higher specificity for APS. Routine testing for anti-DI antibodies in clinical practice can be used for an easy differentiation of subjects carrying clinically meaningful anti-β2GPI antibodies from those individuals with a benign autoantibody profile.

The aim of our study was to determine the significance of the domain I in the anti-β-2-glycoprotein I as a new biomarker for determining thrombotic risk in antiphospholipid syndrome.

We investigate the DI anti-β2GPI on a group of 74 patients with antiphospholipid syndrome diagnosis. All patients have positive antibodies in at least one class ACL and anti-β2GPI antibodies.

We detect DI anti-β2GPI positivity in 21 samples in our group. The thrombotic complications had been observed in 21 from 74 patients. The incidence of thrombotic complications in the total group was established as 28.4%, in comparison to the group DI anti-β2GPI positive with the incidence of thrombotic complications 57%. Performing of assay improved a positive predictive value from 25% pre-test to 68% for patients with positive test.

The new chemiluminescent method for detection of DI anti-β2GPI shows a better compliance with clinical outcome than the actual diagnostic scheme.

Keywords: Anticardiolipin antibodies; Anti-domain I β-2 glycoprotein I antibodies; Antiphospholipid syndrome

Introduction

Antiphospholipid antibodies (APLS) represent a heterogeneous group of antibodies that recognize phospholipids (PL), PL-binding proteins or PL-protein complexes. There is strong evidence that APLS are pathogenic *in-vivo* leading to a large variety of clinical manifestations among which vascular thrombosis and recurrent fetal loss are the most prevalent [1].

In accordance with the revised classification criteria for the antiphospholipid syndrome they are mostly about these manifestations:

- One or more clinical episodes of arterial, venous or small vessel thrombosis in any tissue or organ. Thrombosis must be confirmed by an objective validated criterion (i.e. unequivocal findings of appropriate imaging studies or histopathology). For histopathologic confirmation thrombosis should be present without significant evidence of inflammation in the vessel wall.

- One or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation with normal fetal morphology documented by ultrasound or by direct examination of the fetus; one or more premature births of a morphologically normal neonate before the 34th week of gestation because of eclampsia or severe preeclampsia or recognized features of placental insufficiency; three or more unexplained consecutive spontaneous abortions before the 10th week of gestation with maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded [1].

The presence of these clinical events associated to the detection of APLS in the blood characterizes the antiphospholipid syndrome (APS). Anti-β2GPI antibodies specifically reacting with DI have a particular clinical importance being more commonly detected among patients with APS and other autoimmune diseases. In 1998 the importance of antibodies against DI was described for the first time [2]. The dominant epitope for binding anti-β2GPI antibodies was localized in

DI of β2GPI. The key feature for thrombogenicity of DI is conformational accessibility. In the circular conformation of β2GPI, DI interacts with domain V and the critical epitope is thus hidden. When the β2GPI has an S-shape structure the binding site of epitope in domain I is protected by DIII-IV carbohydrate chains. These residues form a cover over DI and so the binding of antibodies is prevented (Figure 1).
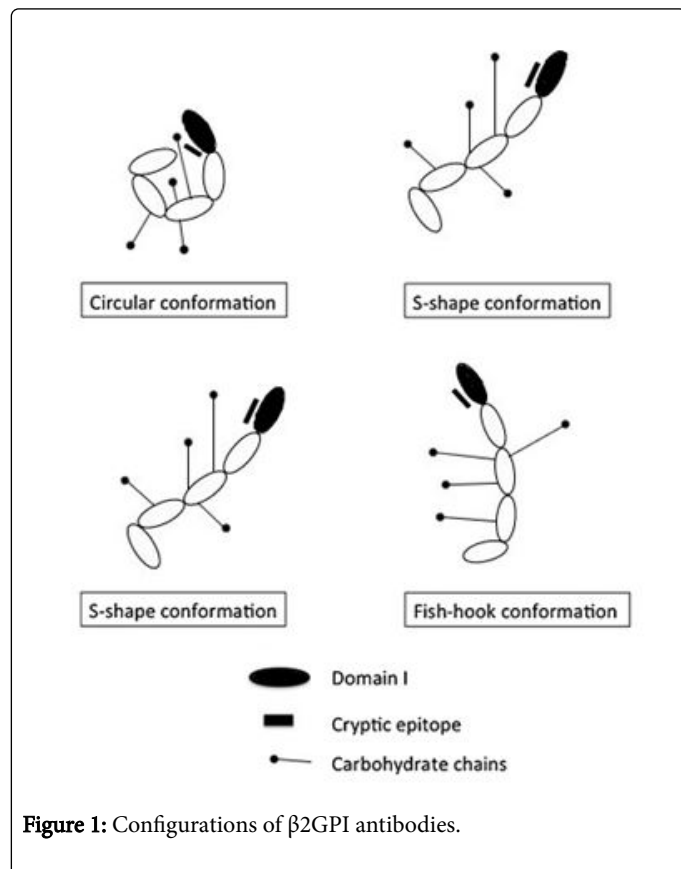

Figure 1: Configurations of β2GPI antibodies.

Evidence of the pathogenicity of anti-DI antibodies comes from both *in-vitro* and *in-vivo* studies. First, anti-DI antibodies were repeatedly observed to induce *in-vitro* prolongation of clotting time [3-8].

More recently, it was revealed that a greater increase in TF activity and significantly larger thrombus were induced by eluted fractions rich in anti-DI antibodies obtained from an antiphospholipid syndrome (APS) patient than by the anti-DI-antibody-poor serum recollected after affinity-purification [9]. Other than the two steps ELISA test using both hydrophilic and hydrophobic plates a few additional ELISA assays have been developed to detect anti-DI antibodies each using different molecular antigenic targets. Recently, a DI anti-β2GPI chemiluminescence immunoassay (CLIA, INOVA Diagnostics, San Diego, USA) has been developed which uses a recombinant DI coupled to paramagnetic beads by use of the BIO-FLASH technology (Biokit, Barcelona, Spain) [10]. The ELISA and CLIA research assays, both developed by INOVA Diagnostics (San Diego, CA, USA) have been directly compared revealing that the two methods have the same specificity but a different sensitivity [11]. The CLIA immunoassay has also been evaluated in comparison to a UK in-house ELISA test; a good agreement between the two tests was observed [12].

The evidence of thrombogenicity anti-DI antibodies comes from both *in-vitro* and *in-vivo* studies. It has been repeatedly observed *in-vitro* prolongation coagulation test with positivity antibodies against DI [3,8].

Recent work also describes a higher expression of TF fractions rich on anti-DI antibodies obtained from patients with APS was repeated and associated higher growth of the thrombus [13].

These findings have led to the formation of the first diagnostic kits to detect the anti-DI antibodies. The first was a two-step ELISA assay with antibodies bound to the hydrophilic and hydrophobic plate for anti-DI antibodies each using a different molecular antigenic target. More recently developed immunological assay based on chemiluminescence β2GPI-DI (CLIA INOVA Diagnostics, San Diego, USA) which uses recombinant DI bound to the paramagnetic beads via the BIO-FLASH technology (BIOKIT, Barcelona, Spain) [10-12].

Mateirals and Methods

The group of patients

This study was conducted on a set of blood samples from 74 patients with detected anticardiolipin antibodies positivity that was sent to our laboratory between January and October 2015. Citrate plasma samples were immediately analyzed or stored at -80°C for CLIA and ELISA.

Blood collection

Blood sampling carried out in a single vacuum tube using a Vacuette® needle (Greiner Bio-One, Vienna, Austria) with a buffered solution containing sodium citrate at a concentration 0.109 mol/L (3.2%). The system ensured blood and anticoagulant mixture at a desired 1:10 ratio. Then the blood was carefully mixed in a test tube then with the tube being gently turned upside down several times and transported to the laboratory. Then the sample was centrifuged two times for 10 minutes at 3000 g the upper layer of the aspirate 0.5 mL of platelet-poor plasma (PPP) and it was frozen and stored at -80°C until CLIA and ELISA was performed. For the actual analysis the sample was thawed in a thermostat at 37°C for 20 minutes.

Lupus anticoagulants

Lupus anticoagulants were detected by two phospholipid dependent test APTT-SP (Werfen, Milano, Italy) and DRVVT (Werfen, Milano, Italy).

Autoantibody assays

ACL and aβ2GPI including DI anti-β2GPI antibodies were measured by CLIA kits (Werfen, Barcelona, Spain)-the assay is currently in use in our lab. The results are expressed in U/mL [14]. The CLIA method was performed with Acustar (Werfen, Barcelona, Spain) a random-access immunoanalyzer uses a two-step immunoassay method based on the principle of chemiluminescence. β2GPI or cardiolipin/β2GPI complex is used to coat magnetic particles and a human anti-IgG or anti-IgM is labeled with conjugate. During the first incubation the specific antibodies presented in the sample in the calibrators or in the controls bind with the solid phase. During the second incubation the conjugate reacted with the antibodies captured on the solid phase after each incubation the materials that has not bounded with the solid phase is removed by suction and repeated washing.

The quantity of marked conjugate bounded to the solid phase is evaluated by chemiluminescent reaction and measured by the light signal. The generated signal measured in RLU (Relative Light Units) is indicative of the concentration of the specific antibodies present in the sample. For ACL IgG or IgM the concentrations of the calibrators are expressed in U/mL (U=units) and calibrated against the "Harris" reference sera. For anti-β2GPI IgG or IgM the concentrations of the calibrators are expressed in U/mL and calibrated against an internal reference standard not further specified by the manufacturer.

Each sample was analyzed in duplicate (calibrators, controls, reference population and patient samples). We determined in-house cut-off values using 50 healthy volunteers with the method of percentiles (99th) [15]. Quality control material provided by the manufacturer was analyzed in every run.

The APS IgM or IgG control set provides a ready to use positive control where we know quantity of ACL or aβ2GPI antibodies and a negative control containing normal human serum. As a result of the positive control imprecision characteristics were evaluated.

Statistical analysis

The positive and negative predictive values (PPV and NPV respectively) are the proportions of positive and negative results in statistics and diagnostic tests that are true positive and true negative results. The PPV and NPV describe the performance of a diagnostic test or other statistical measure. A high result can be interpreted as indicating the accuracy of such a statistic. The PPV and NPV are not intrinsic to the test; they depend also on the prevalence. The PPV can be derived using Bayes' theorem [16].

Results

CLIA methods for assessing Domain I anti-β2GPI were applied on a group of 74 patients with antiphospholipid syndrome. All patients have positive antibodies in at least one class of lupus anticoagulants, ACL and anti-β2GPI antibodies. From the viewpoint of occurrence laboratory parameters for APS were stratification of patients even 20% single positivity, 36% double positivity and 43% triple positivity of tests (Tables 1 and 2).

Parameters	Single positivity	Double positivity	Triple positivity
Domain I negative	14	18	21
Domain I positive	1	9	11

Table 1: Distribution of results for domain I aβ2GPI based on the seriousness of detection antiphospholipid antibodies.

CLIA	ACL IgM	ACL IgG	aβ2GPI IgG	aβ2GPI IgM	Domain I aβ2GPI
Manufacturer	20	20	20	20	20
99th percentile	15.3	13.3	14.2	6.3	3.6

Table 2: The cut-off values for positivity all methods were calculated with the 99th percentile.

The cut-off values for positivity all methods were calculated with the 99th percentile Inter-assay imprecision characteristics were calculated from the results of the commercial positive control material (Table 3).

Coefficients of variation (CV) for the positive control material ranged from 5.4% to 12.7%.

Parameter	ACL IgM	ACL IgG	aβ2GPI IgG	aβ2GPI IgM	Domain I aβ2GPI
CLIA	12.74	10.56	9.04	9.11	5.4

Table 3: The inter-assay imprecision for CLIA method.

The negative control yielded negative results in every run the manufacturer's cut-off is applied. As for in-house cut-off for IgG the normal control sample was above this cut-off.

The clinical manifestations of APS in patients were evaluated as the incidence of thromboembolic disease as deep vein thrombosis (DVT) or pulmonary embolism (PE) based on duplex sonography respectively pulmonary CT angiography. The manifestation of APS in our group of patients was detected in 28% patients.

From the perspective of the clinical manifestation APS there was evaluated the TEN incidences in DVT or PE form. It was based on duplex sonography or rather CT pulmonary angiography (Table 4).

Parameters	APL clinical positivity	APL clinical negativity	Sensitivity
Pre-test	21	53	28% positive prediction value
Domain I negative	9	44	17% positive prediction value
Domain I positive	12	9	57% positive prediction value

Table 4: The sensitivity of test for DI anti-β-2-glycoprotein I.

To evaluate the benefit of antibodies against the domain I to the risk of clinical manifestations of APS there was evaluated PPV in patients with positive or rather negative results of the domain I against APS manifestations of in the entire set.

Discussion

One of the most difficult clinical issues in APS is the lack of specificity of the ACL assays for the diagnosis, leading to the possibility of false-positive diagnoses.

For the reason there were suggested specific laboratory criteria for the APS diagnostics, which defines both phospholipid dependent tests for the determination of lupus anticoagulants and also the methodology for determination of anticardiolipin antibodies [17]. It is not also recommended to conduct any other tests for APS diagnostics [18]. However, the recent publication indicates a possibility of using various subtypes of the anti-β2GPI antibodies to increase determination APS specificity [19].

Increasing evidence suggests the DI is the most relevant epitope targeted by anti-β2GPI antibodies in patients with autoimmune conditions. Anti-DI antibodies have been consistently revealed to be clinically interesting, being significantly associated with both vascular and obstetric APL related events. Our data show that the additional detection of antibodies to domain I to classic panel tests can greatly increase (from 25% to 68%) the positive prediction manifestations of APS.

Recent work described three configurations of β2GPI circulating, J-shaped fish-hook and S-shape. Circulating plasma β2GPI upon binding to suitable anionic surfaces for example to cardiolipin (CL) and other phospholipids opens up to a J-shaped fish-hook configuration with an intermediate S-shape configuration. Only the open structure J-shaped and S-shaped enables to detect the presence of antibodies against the domain I which is in this case accessible for bond. The separate issue is the binding of antibodies from some patients not only to phospholipid and β2GPI but may also be directed to prothrombin, annexin V, protein C and protein S as indicated above.

Despite all the findings it is far too soon to recommend any changes in the antibody assay it appears to complement investigation of β2GPI antibodies specificity against Domain I promising way to increase the specificity of the antibodies investigations. Simultaneously it was not confirmed that the detection of antibodies against domain I is not sufficiently standardized and shows in comparison with the assay for detection of antibodies against the whole molecule lower susceptibility to the APS [20,21]. Although anti-DI antibodies are significantly associated with APS clinical events and with a high-risk APL profile there is still no definite prospective evidence that this test may provide stronger risk factor than anti-β2GPI antibodies for APL related manifestations.

Conclusion

Domain it appears to be the most important epitopes of the anti-β2GPI antibodies in patients with the APS. In view of our results anti-DI antibodies seem to be clinically interesting for manifestations prediction of APS in the form of vascular and obstetric events which are related to APL.

On the strength of these results the complement investigation Domain I appear to be more appropriate for supplement testing of panel than as a substitution of testing anti-β2GPI antibodies. It is mainly about the inadequate comparison of sensitivity and specificity of determination antibodies against Domain I when we compare it with the detection test of antibodies against to a molecule. This could lead to bad APS diagnosis and potential danger for patients.

Although anti-DI antibodies are significantly associated with the clinical manifestations of APS and with a high-risk profile of APL it is not still clearly proved that this test can provide more accurate prediction of risk than the total determination of anti-β2GPI antibodies in APL. However, several pilot studies including our one show that the determination of anti-DI antibodies may enable more direct diagnosis and risk stratification.

As a diagnostic purpose, the anti-DI antibodies are very promising tool for the APS diagnosis. We hope that our work will contribute to a clear definition of diagnostic and prognostic value of anti-DI antibodies. We believe that within a few years testing anti-DI antibodies becomes part of clinical practice.

Acknowledgement

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