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Immunochemical properties and pathological relevance of anti-β_2-glycoprotein I antibodies of different avidity

Urška Žager[1], Špela Irman[1], Mojca Lunder[2], Miha Škarabot[3,4], Igor Muševič[3,4], Vesna Hodnik[5], Gregor Anderluh[5], Saša Čučnik[1], Tanja Kveder[1], Blaž Rozman[1] and **Borut Božič[1,6]**

[1]Department of Rheumatology, University Medical Centre Ljubljana, SI- 1000 Ljubljana, Slovenia
[2]Chair of Pharmaceutical Biology, Faculty of Pharmacy, University of Ljubljana, SI-1000 Ljubljana, Slovenia
[3]Department of Condensed Matter Physics, Jozef Stefan Institute, SI- 1000 Ljubljana, Slovenia
[4]Department of Physics, Faculty of Mathematics and Physics, University of Ljubljana, SI- 1000 Ljubljana, Slovenia
[5]Department of Biology, Biotechnical Faculty, University of Ljubljana, SI- 1000 Ljubljana, Slovenia
[6]Chair for Clinical Biochemistry, Faculty of Pharmacy, University of Ljubljana, SI-1000 Ljubljana, Slovenia

Correspondence to: B. Božič; E-mail: borut.bozic@ffa.uni-lj.si

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Abstract

Despite available treatment, there is still significant morbidity and mortality present among patients with the autoimmune thrombophilic condition termed 'antiphospholipid syndrome' (Espinosa, G. and Cervera, R. 2009. Morbidity and mortality in the antiphospholipid syndrome. Curr. Opin. Pulm. Med. 15:413.). High-avidity (HAv) anti-β_2-glycoprotein I (anti-β_2GPI) antibodies, shown to correlate with thrombotic events in patients, could represent the much needed improved prognostic marker. By studying their effect on crystalline annexin A5 shield on phospholipid surfaces (one of proposed pathogenic mechanisms), with the use of atomic force microscopy, the pathogenic potential of HAv anti-β_2GPI antibodies was confirmed. Furthermore, by using surface plasmon resonance and enzyme-linked immunosorbent assays, unique binding characteristics of HAv antibodies in comparison with low avidity antibodies were established. HAv anti-β_2GPI were confirmed to (i) recognize β_2-glycoprotein I in a solution, (ii) interact predominantly monovalently (much lower dependency on the antigen density) and (iii) form more stable complexes with the antigen. Since enzyme-linked immunosorbent assays currently used in routine diagnostics detect anti-β_2GPI antibodies of unknown avidity, our observations are potentially useful for the development of improved diagnostic tests capable of detecting clinically relevant antibodies.

Keywords: affinity, annexin A5, antiphospholipid antibody, atomic force microscopy, surface plasmon resonance

Introduction

Anti-β_2-glycoprotein I (anti-β_2GPI) antibodies as one of the hallmarks of the antiphospholipid syndrome (APS) significantly correlate with thrombosis and recurrent pregnancy loss (1–4). However, not all patients with elevated anti-β_2GPI show clinical symptoms. This could be explained by heterogeneity of anti-β_2GPI regarding their epitope specificity and their avidity. Such diversity is well expected for an *in vivo* B-cell response, reflected in the production of the anti-β_2GPI of different avidity and the location and nature of epitopes recognized on β_2-glycoprotein I (β_2GPI) (5–7). Heterogeneity of anti-β_2GPI in regard to their avidity has been confirmed by chaotropic ELISA (8, 9). Clinical significance of autoantibodies' avidity has been addressed many times and is still the subject of the ongoing studies. Even 15 years ago, Gharavi and Reiber (10) suggested that high-avidity (HAv) autoantibodies could play a critical role in organ-specific autoimmune disorders, whereas in immune complex-mediated disorders, HAv and low-avidity (LAv) antibodies might be equally pathogenic. Vlachoyiannopoulos *et al.* (9) showed that APS-associated anti-β_2GPI usually exhibit high urea resistance, which is compatible with HAv, while LAv anti-β_2GPI are present in non-APS individuals. According to Reddel and Krilis (11), APS does not appear to be a uniform predilection to thrombosis but, rather, a spectrum of severity that may depend on many factors including antiphospholipid antibodies' avidity. The tendency of HAv anti-β_2GPI in APS patients to be associated with thrombosis has been subsequently reported (8, 12, 13). According to de Laat *et al.* (14), pathologic anti-β_2GPI can be divided into those targeting epitope on domain I (directed against the G40-R43

residues) which highly correlate with thrombosis, and those targeting non-domain I epitopes. Giannakopoulos *et al.* (15) hypothesized that these differences are due to anti-β_2GPI avidity.

Many mechanisms have been proposed to explain the pathogenic function of anti-β_2GPI, one of them being the increased resistance to annexin A5 (ANX A5) (16–19). The anticoagulant properties of ANX A5 are a consequence of ANX A5 crystallization on phospholipid membranes, resulting in a protein lattice over phospholipid surfaces (20)—hindered phospholipid surfaces are blocked and consequently unavailable for coagulation reactions. Recently, Irman *et al.* (21, 22) reported that APS patients-derived antibodies (anti-β_2GPI and anti-ANX A5) interfere with the formation of the ANX A5 shield on phospholipid bilayers which was suggested to accelerate phospholipid-dependent coagulation reactions (23, 24).

Together with the fact that anti-β_2GPI avidity is a rather stable parameter in an individual patient (25), the presence of HAv anti-β_2GPI could very well be considered as a (specific) prognostic marker. Therefore, the aim of the present work was (i) to compare the binding of HAv and LAv polyclonal anti-β_2GPI to β_2GPI under various conditions and (ii) to visualize the pathogenic relevance of HAv anti-β_2GPI on the ANX A5 anticoagulant shield on an *in vitro* model of solid-supported phospholipid bilayers (SPBs). For this purpose, we affinity separated anti-β_2GPI of each individual patient by gradient salt (ionic strength) elution and obtained two anti-β_2GPI fractions: one predominately containing LAv anti-β_2GPI and the other predominately containing HAv anti-β_2GPI.

Methods

Serum samples

Sera of 30 patients with APS and/or systemic lupus erythematosus, positive for anti-β_2GPI IgG, were selected from the sera bank of the Department of Rheumatology, University Medical Centre, Ljubljana, Slovenia. Avidity of anti-β_2GPI IgG was determined using chaotropic ELISA. Two sera were selected based on their avidity, titer and volume availability for the purification of antibodies. Both sera were from patients with primary APS, who suffered initially from venous thrombosis and were positive for IgG anticardiolipin antibodies and IgG anti-β_2GPI. IgG fraction of serum A (sample A) predominately contained HAv anti-β_2GPI. At the time of serum collection, the patient had overt thrombotic manifestation, namely a central nervous system and skin microthromboses, diagnosed as catastrophic APS which later on resulted in the patient's death. IgG fraction of serum B (sample B) contained anti-β_2GPI of heterogeneous avidity. At the time of serum collection, the patient was symptom free regarding APS and had the only thrombotic manifestation several years ago. Two healthy donors' sera without antiphospholipid antibodies (designated C and D) were also selected as negative controls. As positive control, a human chimeric IgG monoclonal anti-β_2GPI antibody (HCAL) (26) was used (Inova Diagnostics Inc., San Diego, CA, USA). The study was approved by the Ethics' Committee of the Slovenian Ministry of Health.

Avidity determination of anti-β_2GPI by chaotropic ELISA

Avidity of anti-β_2GPI was determined by introducing chaotropic conditions in the anti-β_2GPI ELISA during the antibody-binding phase. Sera, sample A and sample B were diluted in PBS, pH 7.4, with 0.05% Tween 20 (0.05% PBST) containing increasing concentrations of NaCl (0.15, 0.25, 0.5, 1, 2 and 4 M) and applied to β_2GPI-coated microtiter plates (High Binding; Costar, Cambridge, MA, USA). The detection system was the same as in the anti-β_2GPI ELISA. Samples in which 70% of the initial binding (at 0.15 M NaCl) was preserved at 0.5 M NaCl were declared as HAv, samples in which the initial binding decreased to and below 25% were declared as LAv, and the samples in between the criteria were declared heterogeneous (8). Sample A was declared as predominantly HAv with 91% of initial binding and sample B as heterogeneous avidity with 46% of initial binding.

Affinity purification of HAv and LAv anti-β_2GPI

Total IgG from selected sera (sample A and sample B) were isolated by affinity chromatography (MabTrapTM Kit; Amersham, GE Healthcare, Little Chalfont, UK), according to the manufacturer's instructions. LAv and HAv anti-β_2GPI were isolated by an in-house β_2GPI affinity column. The column was prepared as described elsewhere (8) by coupling 30 mg of pure human unnicked β_2GPI (27) to 10 ml of CNBr-activated Sepharose 4B (Sigma–Aldrich, St Louis, MO, USA). The unreacted sites on the matrix were blocked with 0.2 M glycine, and the column was equilibrated with PBS with 0.5 M NaCl and 0.1% Tween 20. Sample A and sample B were applied separately to the β_2GPI affinity column and circulated for 90 min at 4°C. After extensive washing with 0.05% PBST, LAv anti-β_2GPI were eluted with 0.5 M NaCl/0.05% PBST and HAv anti-β_2GPI with 0.1 M glycine/4 M NaCl/0.05% PBST, pH 2.5. Eluates were immediately neutralized, dialysed overnight against PBS pH 7.4 and concentrated (Amicon ultra centrifugal unit; Millipore, Billerica, MA, USA). It should be pointed out that each of these fractions is polyclonal and heterogeneous, still containing specificities of different avidity, but in a specific range (lower or higher) of avidity. For atomic force microscopy (AFM) experiments, anti-β_2GPI were dialysed against HEPES-buffered saline (10 mM HEPES, 150 mM NaCl), containing 1.5 mM calcium (HBS-Ca^{2+}), pH 7.5, prepared in water for injections (Braun, Melsungen, Germany). Concentrations and activities of recovered anti-β_2GPI fractions were determined spectrophotometrically (Camspec M501 Single Beam Scanning UV/Visible Spectrophotometer; Camspec Ltd., Cambridge, UK), using the extinction coefficient of 14.0 for the 1% IgG solution and by an in-house anti-β_2GPI ELISA (25).

Influence of β_2GPI density on solid surface on binding of HAv and LAv anti-β_2GPI

Modified anti-β_2GPI ELISA was used. Briefly, polystyrene microtiter plates (High binding; Costar, Cambridge, MA, USA) were coated with various amounts of β_2GPI ranging from 0.3 to 10 ng mm^{-2} and incubated for 2 h at room temperature. Plates were blocked with 1% BSA (Sigma, St Louis, MO, USA) and after 2 h washed with 0.05% PBST. Same dilutions of LAv anti-β_2GPI (from sample B) and HAv

anti-β_2GPI (from sample A) ranging from 45 to 453 pM were applied to the wells for 30 min at room temperature. The percentage of anti-β_2GPI binding was calculated.

Evaluation of monovalent binding of HAv anti-β_2GPI

Fab fragments of HAv anti-β_2GPI from sample B were prepared by papain digestion as described (5). The efficiency of the digestion was confirmed by non-reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (5). The elution of Fab fragments from gel slices was done using an Electro-Eluter (Model 422; Bio-Rad Laboratories, Richmond, VA, USA) according to the manufacturer's instructions. For the evaluation of HAv anti-β_2GPI monovalent binding, different concentrations of high-affinity Fab fragments and HAv anti-β_2GPI ranging from 20 to 1280 pM were tested by ELISA.

Analysis of antibodies' interaction with β_2GPI by surface plasmon resonance

Interaction analyses were performed on Biacore T100 instrument (GE Healthcare, Uppsala, Sweden). β_2GPI was amine coupled to the CM5 Series S sensor chip (GE Healthcare) with *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylpropyl)-carbodiimide, using 10 mM acetate, pH 4.0, as a coupling buffer (GE Healthcare). The immobilization level was ~1000 response units (RU). The running buffer was 0.005% PBST, pH 7.4, and the analysis was performed at 25°C at a flow rate of 10 μl min^{-1}. Seven different concentrations of each anti-β_2GPI fraction in running buffer were prepared, injected over the sensor surface for a period of 3 min and followed by a 3-min dissociation phase. Regeneration of the sensor surface between sample exposures was done with a short pulse of 10 mM NaOH. Obtained sensorgrams were corrected by double subtracting the signal obtained on a reference surface and the signal of the running buffer. For evaluation of relative binding affinity of polyclonal anti-β_2GPI, a procedure described by Metzger *et al.* (28) was used.

Binding of HAv and LAv anti-β_2GPI to native β_2GPI in solution

Two dilutions (0.45 and 0.06 nM) of LAv anti-β_2GPI (from sample B) and HAv anti-β_2GPI (from sample A) and HCAL were prepared in 0.05% PBST and mixed with purified β_2GPI (8). The final concentrations of β_2GPI were 8, 5, 2, 1, 0.5, 0.2, 0.1 and 0 μM. After 2 h incubation, at room temperature, the extent of anti-β_2GPI binding to native β_2GPI in solution was determined by anti-β_2GPI ELISA (25).

Effect of HAv anti-β_2GPI on ANX A5 anticoagulant crystal shield on an artificial membrane analysed by atomic force microscopy

The effect of HAv anti-β_2GPI on incompletely crystallized ANX A5 on SPBs was observed and studied with AFM in a liquid environment using a Nanoscope IIIa-MultiMode AFM (Digital Instruments, Santa Barbara, CA, USA) equipped with the E (15 μm) scanner, as previously described (21).

Briefly, SPBs composed of 30% (w/w) of L-α-phosphatidylserine and 70% of L-α-phosphatidylcholine (Sigma–Aldrich) were prepared on mica (21). When the presence of SPB was confirmed, ANX A5 (10 mg l^{-1} in HBS-Ca^{2+}) isolated from human placenta (Sigma–Aldrich) was injected into the fluid cell of the AFM. After formation of the ANX A5 crystalline shield incompletely covering the SPB, β_2GPI (0.15 g l^{-1} in HBS-Ca^{2+}) and HAv anti-β_2GPI from sample A (0.4 g l^{-1} in HBS-Ca^{2+}) or control antibodies (from serum C, 10 g l^{-1} in HBS-Ca^{2+}) were added to the ANX A5 solution covering the SPB. The effect of the antigen–antibody pair on incompletely crystallized ANX A5 on SPBs was measured for 60 min.

Results

Influence of β_2GPI density on solid surface on binding of HAv and LAv anti-β_2GPI

LAv and HAv anti-β_2GPI of equal concentrations were applied to decreasing antigen densities immobilized on microtiter plates. It was observed that LAv anti-β_2GPI were far more dependent of β_2GPI density than HAv anti-β_2GPI. On high-density β_2GPI (10 ng mm^{-2}), both fractions exhibited comparable degrees of binding (Fig. 1, left), whereas on low-density β_2GPI, a distinct difference in binding was observed between the two groups. While the binding of HAv anti-β_2GPI was still effective, though diminished to some degree due to a lower amount of the antigen, the binding of LAv fraction was completely abolished (Fig. 1, right). To achieve an effective binding of LAv anti-β_2GPI and HAv anti-β_2GPI, densities of 2.5 ng mm^{-2} and 0.6 ng mm^{-2} of β_2GPI, respectively, were required, pointing to a greater tendency of LAv anti-β_2GPI to bivalent binding, feasible above the threshold of the antigen density.

Evaluation of monovalent binding of HAv anti-β_2GPI

High-affinity Fab fragments exhibited concentration-dependent binding to immobilized β_2GPI. At low concentrations, the binding of HAv anti-β_2GPI (bivalent molecules) and high-affinity Fab fragments (monovalent molecules) to β_2GPI were almost

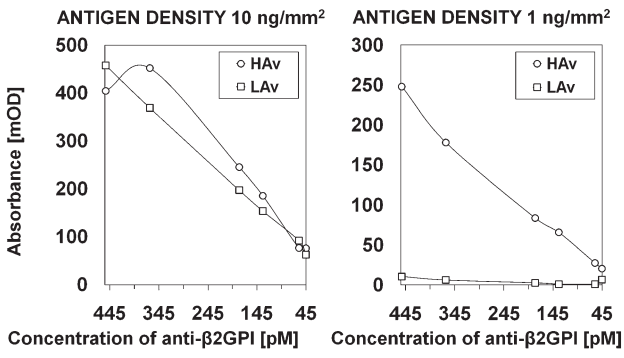

Fig. 1. Binding of LAv and HAv antibodies against β_2GPI (anti-β_2GPI) to high and low density of immobilized β_2GPI. Binding of various concentrations of LAv (open squares) and HAv (open circles) anti-β_2GPI to high (10 ng mm^{-2}) and low (1 ng mm^{-2}) density of β_2GPI absorbed on microtitre plates is presented. On high density of β_2GPI, the binding of both HAv and LAv anti-β_2GPI was effective (left), whereas on low density of β_2GPI, the binding of LAv anti-β_2GPI was abolished and only the binding of HAv anti-β_2GPI was effective (right). Results presented as A [mOD] at 405 nm values are the average of duplicate measurements and the variability was <11%.

equal (Fig. 2). With increasing concentrations, the binding of monovalent high-affinity Fab fragments and bivalent HAv anti-β_2GPI differed up to 32%. Our results confirmed that considerable amounts of HAv anti-β_2GPI bound monovalently to the antigen.

Surface plasmon resonance analysis of antibodies' interaction with immobilized β_2GPI

A biosensor analysis was used to observe the binding characteristics of polyclonal LAv and HAv anti-β_2GPI in real time. Monoclonal anti-β_2GPI (HCAL) was used to evaluate the stability of a β_2GPI-coated surface (1000 RU corresponding to 1 ng mm^{-2} (29)) and to determine the optimal experimental conditions. The reproducibility of responses confirmed the relevance of regeneration protocol to disrupt antibody–antigen interactions without compromising the activity of the latter (data not shown). A healthy donor's IgG (IgG D) represented the negative control.

For biosensor analysis, the amount of active polyclonal anti-β_2GPI was determined by ELISA. The highest possible concentrations together with 1:1 serial dilutions of active anti-β_2GPI obtained by several isolations were used: LAv anti-β_2GPI from sample A and sample B at 156 and 110 nM and HAv anti-β_2GPI from sample A and sample B at 308 and 122 nM, respectively. The responses of LAv and HAv anti-β_2GPI were concentration dependent, as shown in Fig. 3. IgG D did not bind to β_2GPI, as expected (Fig. 3). The shapes of interaction curves for LAv and HAv anti-β_2GPI (for sample A and sample B) differed noticeably. It is evident from Fig. 4 that sample A contained a more homogeneous population of antibodies in terms of avidity (antibodies of more uniform avidity), whereas sample B contained an extremely heterogeneous population of antibodies where difference in avidity was more pronounced.

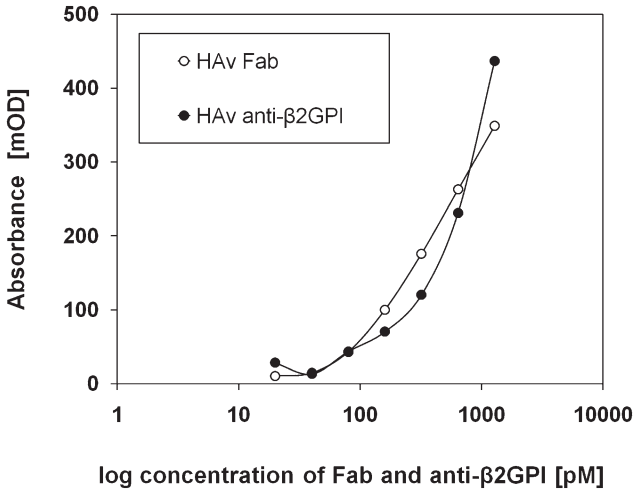

Fig. 2. Evaluation of monovalent binding of HAv antibodies against β_2GPI (anti-β_2GPI) to the immobilized β_2GPI. Binding of various concentrations of high-affinity anti-β_2GPI Fab fragments (monovalent molecules) and of HAv anti-β_2GPI (bivalent molecules) to β_2GPI absorbed on microtitre plates is presented. High-affinity Fab and HAv anti-β_2GPI exhibited similar extent of binding. Results presented as A [mOD] at 405 nm are the average of duplicate measurements and the variability was <10%.

The amount of bimolecular β_2GPI–anti-β_2GPI complexes was determined by measuring the amplitude of the surface plasmon resonance (SPR) signal 176 s after sample injection. The HAv fraction from sample A (154 nM) gave a signal of 501 RU, while for the LAv fraction (156 nM), a lower signal of 442 RU was determined. For sample B, the same yet more pronounced trend was observed: 759 RU for HAv fraction (122 nM) and 284 RU for LAv fraction (110 nM). Results indicated that the polyclonal HAv anti-β_2GPI fraction contained a higher amount of antibodies capable of binding to covalently immobilized β_2GPI at a density of 1 ng mm^{-2} than LAv fraction. Furthermore, the stability of bimolecular β_2GPI–anti-β_2GPI complexes was determined by a decrease in the SPR signal 100 s after the outset of the dissociation period

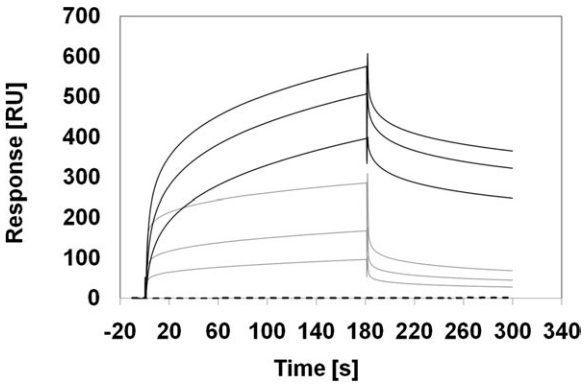

Fig. 3. Evaluation of HAv, LAv antibodies against β_2GPI (anti-β_2GPI) and healthy donor's IgG interactions with the immobilized β_2GPI by SPR. The HAv anti-β_2GPI at titers 308, 154 and 77 nM (black line sensorgrams starting from the top), LAv anti-β_2GPI at 110, 55 and 27.5 nM (grey line sensorgrams) and healthy donor's IgG at 465 and 232.5 nM (dashed line sensorgram at the bottom), respectively, were applied to chip-immobilized β_2GPI. The concentration-dependent binding of HAv and LAv anti-β_2GPI to β2GPI immobilized on SPR chip is presented. Healthy donor's IgG (negative control) did not bind to β_2GPI. Results are presented as RU.

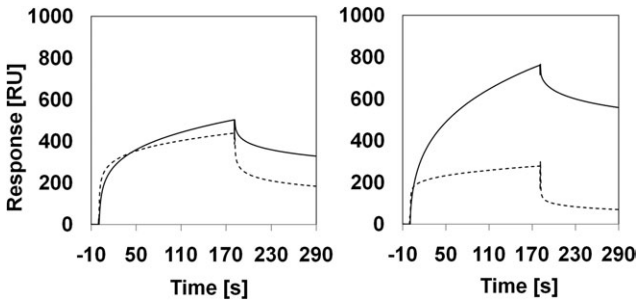

Fig. 4. The comparison of sensorgrams presenting the interaction of LAv and HAv antibodies against β_2GPI (anti-β_2GPI) from sample A and sample B with immobilized β_2GPI. HAv anti-β_2GPI of both samples (unbroken lines) formed larger amount of complexes with β_2GPI (determined by the amplitude of SPR signals at 176 s) and exhibited greater stability of complexes (determined by decrease of SPR signals at 280 s of dissociation phase) than LAv anti-β_2GPI (dashed lines). Left: LAv (dashed line) and HAv (unbroken line) anti-β_2GPI from sample A at 156 and 154 nM; right: LAv (dashed line) and HAv (unbroken line) anti-β_2GPI from sample B at 110 and 122 nM. Results are presented as RU.

(designated as a percentage of dissociation). Dissociation of LAv anti-β_2GPI, 54.3 ± 2.8% (sample A) and 71.9 ± 2.4% (sample B) was significantly faster than of HAv anti-β_2GPI, calculated as 37.8 ± 3.1% (sample A) and 28.2 ± 3.4% (sample B) suggesting greater stability of bimolecular β_2GPI–HAv anti-β_2GPI complexes.

Binding of HAv and LAv anti-β2GPI to native β2GPI in a solution

To evaluate the binding of LAv and HAv anti-β_2GPI to β_2GPI in a solution, inhibition experiments were performed. Both fractions of anti-β_2GPI and HCAL were tested at two concentrations: one corresponding to a low-positive (0.06 nM) and the other to a high-positive ELISA titer (0.45 nM). Antibody solutions were incubated with various concentrations of β_2GPI in a solution. Binding of anti-β_2GPI in an ELISA was inhibited by a concentration-dependent manner by β_2GPI in a solution. HCAL exhibited an average binding to β_2GPI in a solution, similar to that reported by Ambrozic *et al.* (30). Such a result was expected, considering that HCAL is a chimeric antibody containing murine complementarity determining regions and considerable avidity of murine monoclonals (31). Results indicate that the binding of HAv anti-β_2GPI to β_2GPI in a solution was far more pronounced than of LAv anti-β_2GPI. The binding of HCAL to β_2GPI in a solution was higher than the binding of LAv anti-β_2GPI and lower than the binding of HAv anti-β_2GPI. A detectable decrease in ELISA signal of HAv anti-β_2GPI was observed at 0.1 μM of β_2GPI, regardless of the antibody concentration. HAv anti-β_2GPI inhibition of 50% was achieved using physiological concentrations of β_2GPI; meaning 4 μM at a high and 1.2 μM at a low antibody titer (Fig. 5). For a notable decrease in ELISA signal of LAv anti-β_2GPI, 5–10 times higher concentrations of β_2GPI were required than for HAv anti-β_2GPI. The latter also exhibited a far steeper β_2GPI concentration-dependent decrease of ELISA signal than the former. With the highest amount of β_2GPI (8 μM) and the antigen versus antibody molar ratio 10^5:1, only 33% inhibition of LAv anti-β_2GPI was achieved (Fig. 5).

Influence of HAv anti-β2GPI on ANX A5 anticoagulant crystal shield on an artificial membrane

In order to simulate the physiological conditions of the activated cell surface, SPBs from naturally derived phospholipids were prepared on mica (thicknesses of SPBs were 3.6 ± 0.4 nm) and ANX A5 was crystallized over SPBs (thicknesses of crystalline ANX A5 layer over SPBs were 2.6 ± 0.2 nm) (in detail in ref. (21)). When β_2GPI and HAv anti-β_2GPI were added to incompletely crystallized ANX A5 on SPB, the growth of β_2GPI–HAv anti-β_2GPI complexes (14–16 nm higher than the phospholipid surface) was detected (Fig. 6).

HAv anti-β_2GPI alone did not bind to SPB or ANX A5 and had no effect on ANX A5 crystalline lattice structure. For control antibodies IgG C (isolated from a healthy donor without anti-β_2GPI), no binding of antibodies to β_2GPI or effect on crystalline ANX A5 was detected. The order of addition of reagents into the AFM fluid cell to SPBs had no effect on the result.

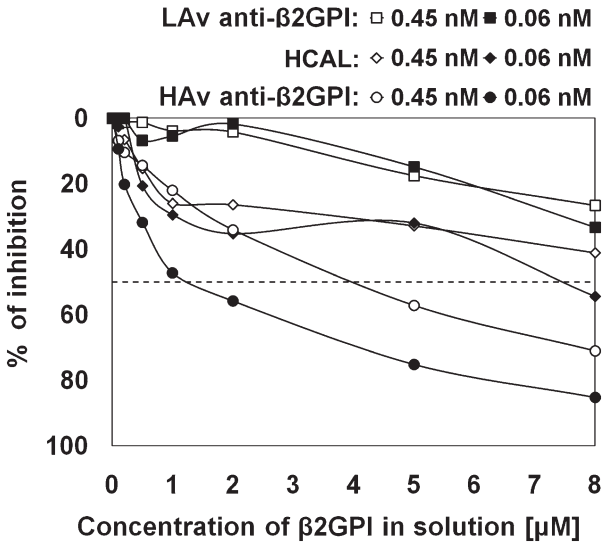

Fig. 5. Inhibition of binding of LAv, HAv and monoclonal (HCAL) antibodies against β_2GPI (anti-β_2GPI) to β_2GPI absorbed on microtitre plates by native β_2GPI in solution. Two dilutions of LAv anti-β_2GPI (open squares 0.45 nM and closed squares 0.06 nM), HAv anti-β_2GPI (open circles 0.45 nM and closed circles 0.06 nM) and monoclonal anti-β_2GPI (HCAL) (open triangles 0.45 nM and closed triangles 0.06 nM) were incubated with various concentrations of native β_2GPI in solution. HAv anti-β_2GPI exhibited considerably higher binding to β_2GPI in solution than LAv anti-β_2GPI. The HCAL also bound to β_2GPI in solution and is shown for comparison. Results are expressed as the percentage of inhibition ((1 − A inhibited/A uninhibited) × 100). Values are the average of duplicate measurements and the cumulative variability was <15%.

Discussion

Isolation and characterization of HAv and LAv anti-β2GPI

Anti-β_2GPI are the main pathogenic players in APS. Reports demonstrating the clinical relevance of HAv anti-β_2GPI prompted us to better characterize binding and functional features of HAv anti-β_2GPI. Some features, however, may not be evident when dealing with a patient's entire polyclonal anti-β_2GPI fraction which contains antibodies of heterogeneous avidity. Therefore, we separated the anti-β_2GPI of each patient (HAv sample A and heterogeneous avidity sample B) by affinity chromatography using gradient elution of increasing ionic strength and obtained fractions containing predominately HAv and fractions containing predominately LAv anti-β_2GPI.

The evaluation of antigen density dependence and binding characteristics under flow conditions revealed some unique features of HAv and LAv anti-β_2GPI. The antigen density influenced bindings of both fractions; however, the influence was far less pronounced for HAv anti-β_2GPI. This indicated that LAv anti-β_2GPI require bivalent interactions with the antigen, whereas the binding of HAv anti-β_2GPI to β_2GPI was predominately monovalent and therefore only slightly influenced by the antigen density, as previously suggested (5). The latter was also confirmed by only slight differences observed in the binding of bivalent (IgG) and monovalent (Fab) high-affinity anti-β_2GPI molecules to β_2GPI immobilized on a microtiter plate.

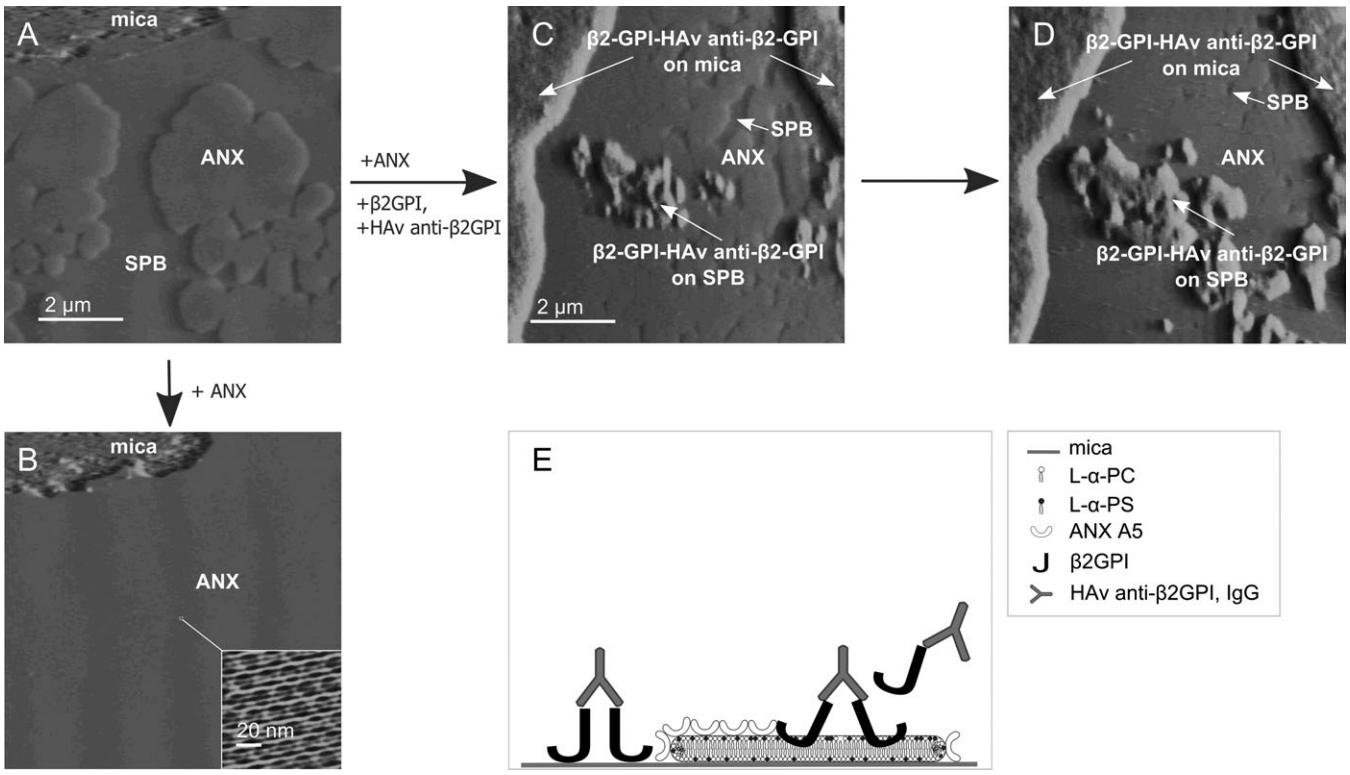

Fig. 6. AFM deflection images and a scheme of the effect of β_2GPI and HAv antibodies against β_2GPI (HAv anti-β_2GPI) on crystalline annexin A5 (ANX A5) on SPBs. (A) Image of mica and crystalline ANX A5 (10 mg l^{-1} in HBSCa2+) not completely covering the SPB; (B) after 15 min of incubating the ANX A5 solution (10 mg l^{-1} in HBS-Ca2+) over the SPB crystalline ANX A5 completely covered the SPB. The right lower corner shows 100 × 100 nm^2 ANX A5 crystalline structure where p6 crystal form can be observed; (C) however, if β_2GPI (0.15 g l^{-1} in HBS-Ca2+) and HAv anti-β_2GPI isolated from sample A (0.4 g l^{-1} in HBS-Ca2+) were added to the ANX A5 (10 mg l^{-1} in HBS-Ca2+) solution covering the incomplete crystalline ANX A5 layer over the SPB, the complexes of β_2GPI and HAv anti-β_2GPI on SPB were measured as well. Antibodies and antigen formed complexes with apparently sufficient affinity to bind to SPB in the presence of crystalline ANX A5. The complexes of β_2GPI and HAv anti-β_2GPI bound to exposed mica surface as well; (D) image of further lateral growth of β_2GPI-HAv anti-β_2GPI patches on SPB in the presence of incompletely crystallized ANX A5 on SPB is shown; (E) scheme of binding complexes of β_2GPI and HAv anti-β_2GPI on mica and to SPB in the presence of incompletely crystallized ANX A5.

Biosensor analysis, performed at lower antigen density than in the ELISA, further confirmed the distinct influence of antigen density on the binding of LAv antibodies. Despite identical concentrations of antibodies, the HAv anti-β_2GPI formed a larger amount of bimolecular antibody–antigen complexes than the LAv anti-β_2GPI, as revealed by the amplitude of the SPR signal (Fig. 4). HAv anti-β_2GPI also formed more stable bimolecular antibody–antigen complexes with β_2GPI than LAv anti-β_2GPI. Analysis of SPR sensorgrams showed slower dissociation of β_2GPI–HAv anti-β_2GPI than of β_2GPI–LAv anti-β_2GPI complexes.

Unexpectedly, the SPR analysis also revealed an interesting difference in the anti-β_2GPI profiles of the two samples being studied: a homogeneous avidity of sample A anti-β_2GPI and an extremely heterogeneous avidity of sample B anti-β_2GPI. Considering the severity of patients' disease manifestations, it was reasonable to assume that patients with homogeneous HAv anti-β_2GPI profile were more prone to develop severe forms of APS than those with a more heterogeneous profile. We can speculate that patient A (compared with patient B) with predominant HAv anti-β_2GPI antibodies had a more aggressive thrombophilic serologic profile resulting in catastrophic APS, from which he later succumbed.

Conformational change of native β_2GPI induced under the influence of HAv anti-β_2GPI

It has been recently reported that β_2GPI can exist in two conformations: a circular plasma conformation and an active open conformation (31). According to the authors, only the murine monoclonal antibodies, which have adequate (high) affinity for β_2GPI's domain I to interrupt the hydrophilic interaction between domains I and V, recognized the circular conformation. The (low affinity) patients' anti-β_2GPI, however, only recognized the open conformation (31). On the contrary, our results show that the patient's HAv anti-β_2GPI contained a considerable amount of antibodies that recognized the circular confirmation since they exhibited moderate affinity towards soluble β_2GPI. About 50% inhibition of their binding to immobilized β_2GPI was easily achieved with physiological concentrations of β_2GPI (32). On the other hand, the binding of LAv anti-β_2GPI to the antigen in a solution was rather weak, as already reported (33–35) and indicated their incapacity to interrupt the bond between domains I and V. However, our findings could also indicate that HAv anti-β_2GPI bound to native epitopes on β_2GPI, whereas the binding of LAv anti-β_2GPI depended on epitopes exposed at the surface of the immobilized antigen.

Impaired formation of ANX A5 anticoagulant crystal shield on an artificial membrane under the influence of HAv anti-β_2GPI

Since Irman *et al.* (21) reported that anti-β_2GPI affect only the ANX A5 shield incompletely covering SPBs, the functional features of HAv anti-β_2GPI were evaluated through their effects on incomplete ANX A5 shield. The effects of HAv anti-β_2GPI in the presence of β_2GPI were visualized and evaluated by imaging with AFM. Vertical heights of the ANX A5 crystalline layer and supramolecular assemblies of β_2GPI on SPBs were in agreement with previous studies (20–22,36–39).

When β_2GPI and HAv anti-β_2GPI were applied to incompletely crystallized ANX A5 on SPBs, the growth of β_2GPI–HAv anti-β_2GPI complexes on the exposed phospholipid surfaces was detected. This effect prevented the formation of the anticoagulant ANX A5 shield *in vitro*. We suggest that HAv anti-β_2GPI were able to dimerize β_2GPI and via immune complexation to multimerize the protein. These multimerized β_2GPI–HAv anti-β_2GPI complexes had much higher affinity for a phospholipid surface than the antigen alone, were adequately stable (as established above) and were therefore able to compete with ANX A5 for the exposed binding sites on negatively charged phospholipid surfaces. The results were in agreement with the previously suggested secondary pathogenic role of anti-β_2GPI, where preliminary endothelial damage or deficiency in ANX A5 synthesis or function is required for the development of thrombotic events (21).

In the present study, HAv anti-β_2GPI were separated from LAv anti-β_2GPI and differences regarding their binding characteristics were established. In addition to HAv anti-β_2GPI's predominant monovalent binding and ability to form stronger complexes with β_2GPI than LAv anti-β_2GPI, the former were also shown to recognize and bind β_2GPI in a solution. In our study, the pathogenic role of HAv anti-β_2GPI in APS-associated features was demonstrated by their ability to interfere with the formation of the anticoagulant ANX A5 shield. HAv anti-β_2GPI were suggested to be clinically relevant due to their stronger correlation with thrombotic events in patients. As suggested previously (8), we continue to demonstrate the clinical importance of HAv anti-β_2GPI, showing that the homogeneous HAv serum profile of anti-β_2GPI present in one of our patients led to catastrophic thrombotic events.

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