Human Monoclonal Antiphospholipid Antibodies Disrupt the Annexin A5 Anticoagulant Crystal Shield on Phospholipid Bilayers

Evidence from Atomic Force Microscopy and Functional Assay

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The antiphospholipid (aPL) syndrome is an autoimmune condition that is marked by recurrent pregnancy losses and/or systemic vascular thrombosis in patients who have antibodies against phospholipid/co-factor complexes. The mechanism(s) for pregnancy losses and thrombosis in this condition is (are) not known. Annexin A5 is a potent anticoagulant protein, expressed by placental trophoblasts and endothelial cells, that crystallizes over anionic phospholipids, shielding them from availability for coagulation reactions. We previously presented data supporting the hypothesis that aPL antibody-mediated disruption of the anticoagulant annexin A5 shield could be a thrombogenic mechanism in the aPL syndrome. However, this has remained a subject of controversy. We therefore used atomic force microscopy, a method previously used to study the crystallization of annexin A5, to image the effects of monoclonal human aPL antibodies on the crystal structure of the protein over phospholipid bilayers. In the presence of the aPL monoclonal antibodies (mAbs) and β_2 -GPI, the major aPL co-factor, structures presumed to be aPL mAb-antigen complexes were associated with varying degrees of disruption to the annexin A5 crystallization pattern over the bilayer. In addition, measurements of prothrombinase activity on the phospholipid bilayers showed that the aPL mAbs reduced

the anti-coagulant effect of annexin A5 and promoted thrombin generation. These data provide morphological evidence that support the hypothesis that aPL antibodies can disrupt annexin A5 binding to phospholipid membranes and permit increased generation of thrombin. The aPL antibody-mediated disruption of the annexin A5 anticoagulant shield may be an important prothrombotic mechanism in the aPL syndrome. (*Am J Pathol* 2003, 163:1193–1200)

The antiphospholipid (aPL) syndrome is an autoimmune disorder marked by recurrent pregnancy losses and vascular thrombosis.^{1,2} Several mechanism(s) for thrombosis in the aPL syndrome have been proposed, however, the pathophysiology of this condition has remained elusive.³ Annexin A5 is a potent phospholipid-binding anticoagulant protein previously known by other names including placental anticoagulant protein I^{4,5} and vascular anticoagulant α^6 (see Kim and Hajjar⁷ for a recent review on the annexin family of proteins). The protein forms two-dimensional (2-D) crystal lattices over anionic phospholipid surfaces^{8–10} and shields the phospholipid from availability for phospholipid-dependent coagulation reactions.¹¹

We previously proposed the hypothesis that aPL antibodies may promote pregnancy losses and thrombosis by disrupting the annexin A5 anticoagulant shield.¹² The aPL antibody-mediated reduction of annexin A5 has been demonstrated by us via ellipsometry,¹³ enzyme-linked immunosorbent assay,^{13,14} and by others using flow cytometry¹⁵ to be caused by displacement of the

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Table 1. Effects of the Human aPL mAbs on Annexin A5 Anticoagulant Activity

	Control IgG1	Control IgG3	aPL IS3	aPL CL1	aPL CL15
Immunological classification					
γ Subclass	$\gamma 1$	$\gamma 3$	$\gamma 3$	$\gamma 3$	$\gamma 3$
Light chain	κ	κ	κ	λ	κ
Thrombin generation (pmol/minute)					
Without β_2 -GPI	224	200	150	705	537
With β_2 -GPI	338	219	1376	1072	1031

annexin by the antibodies. However, this has been a subject of controversy since one group, using ellipsometry, asserted that their data “unambiguously” showed that aPL antibodies are unable to displace annexin V from procoagulant membranes.¹⁶ It would, therefore, be appropriate to use a more direct method to determine whether such displacement could occur. Because the binding of annexin A5 on phospholipid bilayers can be directly imaged by atomic force microscopy (AFM),¹⁰ and because the surface topographies of the crystal forms that bind to the phospholipid bilayers have been characterized,¹⁷ we applied this method to investigate the effects of previously characterized monoclonal human aPL antibodies¹⁸ on the crystal structure of annexin A5. We provide herein the first images of the effects on annexin A5 binding to phospholipid bilayers. We further characterized the aPL monoclonal antibodies (mAbs) by measuring their effects on annexin A5 anticoagulant activity. We found that aPL antibodies can indeed disrupt the crystallization of annexin A5 on phospholipid bilayers and that these antibodies can also reverse the potent anticoagulant effect of this protein.

Materials and Methods

Preparation and Characterization of Human mAbs

Three aPL mAb IgGs designated IS3, CL1, and CL15, whose characteristics were previously described¹⁹ (IgG subclasses and light chains are shown in Results and Table 1), were generated from the peripheral blood mononuclear cells of patients with the aPL syndrome and were purified by affinity columns as previously described.¹⁹ The characteristics of these mAbs have been previously described.¹⁹ By enzyme-linked immunosorbent assay, CL1 and 15 reacted more strongly against human β_2 -GPI than did IS3, which also recognized the protein; interestingly, all three mAbs displayed weak reactivity against cardiolipin alone. Two human mAbs (Sigma, St. Louis, MO), from patients with monoclonal gammopathies were used as controls.

Proteins

Annexin A5 was purified from human placentas according to the method of Funakoshi and colleagues.⁴ The protein was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a previously characterized, affinity-purified monospecific rabbit anti-annexin

A5 IgG, as described.^{20,21} Human β_2 -GPI was purified as previously described.²² Human factor Xa, bovine factor Va, and human prothrombin were generous gifts from Dr. Yale Nemerson (Division of Thrombosis, Mount Sinai School of Medicine, New York, NY).

AFM Studies

Two types of experiments were performed by AFM. For one type, which will be referred to as “dynamic imaging” experiments, the preparations were continuously observed using tapping mode in buffer with a Digital Instruments BioScope (Digital Instruments, Santa Barbara, CA) as the various components were added (see below). Standard 100- μ m narrow-legged, oxide-sharpened pyramidal-tipped silicon nitride probes (Advanced Surface Microscopy, Inc., Indianapolis, IN) were used in tapping mode with an oscillating frequency of 7.8 to 8.0 kHz as previously described.^{23,24} The second type of experiments, which will be referred to as “endpoint-imaging” experiments, were done using single observations that were based on a time course that was established for the formation of the continuous phospholipid bilayer and for the formation of the subsequent structures. These endpoint-imaging experiments enabled the bulk preparation of several slides with preformed annexin A5 crystal layer on phospholipid for subsequent antibody exposure experiments. Moreover, the endpoint-imaging experiments enabled us to ascertain whether continuous interaction between the scanning tip and the specimen produced any artifactual effect on the planar phospholipid or annexin A5 structure. All experiments were performed at room temperature. A phospholipid vesicle mixture of 30% 1,2-dioleoyl-sn-glycero-3-phospho-L-serine and 70% 1,2-dioleoyl-sn-glycero-3-phosphocholine (PS/PC) (Avanti Polar-Phospholipids, Inc., Alabaster, AL) was dried under nitrogen and dissolved in HEPES-buffered saline (HBS) (0.01 mol/L HEPES, 0.14 mol/L NaCl, pH 7.5) containing 1 mmol/L CaCl_2 . The mixture was extruded through a polycarbonate filter (pore size, 100 nm; Poretics, Livermore, CA). Freshly cleaved 1.2-cm mica disks (Ashville-Schoonmaker Mica Co., Newport News, VA) were covered with 200 μ l of the phospholipid mixture at a final concentration of 0.6 mmol/L in HBS containing 1.25 mmol/L CaCl_2 and incubated for 60 minutes. Care was taken to prevent oxidation of the forming planar phospholipid by repeated aspiration and rinsing with buffer, ensuring that the phospholipid was always covered by at least 100 μ l of fresh buffer. In the dynamic imaging experiments, this preparation was next examined by AFM

to confirm the presence of a continuous planar phospholipid bilayer. When a continuous surface that was free of defects was observed, the area was then further analyzed by AFM with force calibration plots to establish the presence of a soft surface overlaying the mica. The presence of the phospholipid bilayer was confirmed by a change in the force calibration plot from a sharp-pointed deflection seen with the mica surface alone, to a sloping rounded surface indicative of probe interaction with a soft surface.²⁵ To study the effects of aPL mAbs on phospholipid-bound annexin A5, annexin A5 was added to a final concentration of 40 $\mu\text{g/ml}$ to the buffer covering the phospholipid bilayer on mica surface after pilot studies had shown that this concentration was necessary to obtain complete coverage of the planar phospholipid bilayer by the annexin A5 2-D crystal layer within an hour. We, therefore, waited at least 60 minutes after the addition of annexin A5 before adding further reagents. β_2 -GPI (15 $\mu\text{g/ml}$) and aPL or control mAbs (80 $\mu\text{g/ml}$) were then added and incubated for a further 60 minutes, while continuously imaging with the AFM. In a further set of experiments, we tested the ability of the annexin A5 2-D crystal layer to form on a planar phospholipid bilayer that had been pretreated with antibodies and co-factor. Planar phospholipid bilayers were formed on freshly cleaved mica disks as described above. The bilayers were then incubated with a mixture of β_2 -GPI (15 $\mu\text{g/ml}$) co-factor and either a specific monoclonal aPL antibody, or a control nonspecific monoclonal human IgG for at least 60 minutes. Annexin A5 was then added to the preparation and imaged for at least 60 minutes.

Control Experiments for AFM

A series of control AFM experiments were performed as follows: 1) annexin A5 on planar phospholipid bilayer was incubated with β_2 -GPI (15 $\mu\text{g/ml}$) without addition of antibody, and imaged for 60 minutes; 2) annexin A5 on planar phospholipid bilayer was incubated with aPL monoclonal antibodies in the absence of β_2 -GPI, and imaged for at least 60 minutes; 3) annexin A5 on planar phospholipid bilayer was incubated with aPL monoclonal antibodies for 60 minutes, followed by addition of β_2 -GPI, and further imaging for at least 60 minutes; 4) annexin A5 on planar phospholipid bilayer was incubated with two control non-aPL human mAbs, IgG1 and IgG3, with β_2 -GPI; and 5) β_2 -GPI on planar phospholipid bilayer, without annexin A5, followed by addition of aPL monoclonal antibodies after 60 minutes.

Prothrombinase Activity Studies

The effects of the aPL mAbs on annexin A5 anticoagulant activity were determined as previously described,²⁶ with minor modifications. A phospholipid mixture consisting of 30% PS and 70% PC was prepared as described above. The vesicles (final concentration, 50 $\mu\text{mol/L}$) were applied to reflective silicon slides in a container containing stirring HBS and 1.25 mmol/L of CaCl_2 . The slides were transferred into an ellipsometer cuvette containing 1 ml of

stirring HBS with 1.25 mmol/L calcium and 0.025% bovine serum albumin. Annexin A5 (5 $\mu\text{g/ml}$) was added to the cuvette. After the protein adsorption, as determined by ellipsometry, had reached equilibrium, each of the aPL or control mAbs (100 $\mu\text{g/ml}$), together with or without β_2 -GPI (30 $\mu\text{g/ml}$), was then added to the cuvette. After adsorptions had reached equilibrium, the slides were flushed with the buffer and the prothrombinase reactants [factor Xa (final concentration, 0.5 nmol/L), factor Va (1 nmol/L), and prothrombin (25 nmol/L)] were added to the cuvette. The enzyme mixture was serially aliquoted (50 $\mu\text{l/well}$) into a microtiter plate filled with 100 $\mu\text{l/well}$ of bicine buffer (50 mmol/L bicine, 0.1 mol/L NaCl, 0.1% bovine serum albumin, 25 mmol/L ethylenediaminetetraacetic acid, pH 8.5). Chromogenic substrate S-2238 (Chromogenix, Mölndal, Sweden) was then added (0.57 mmol/L). The generation of thrombin was measured using a THERMOMax kinetic microplate reader (Molecular Devices, Menlo Park, CA) at 405-nm and 490-nm dual beam at 35°C.

Results

AFM Studies

We used AFM to investigate the structural effect of the aPL mAbs on annexin A5 2-D crystals that had been preformed on a planar phospholipid bilayer. Also, we studied the effects of the mAbs on the subsequent binding of annexin A5. After incubation of cleaved mica with PS/PC vesicles for 60 minutes at room temperature, the surface of the mica was covered with a homogeneous planar layer of phospholipid. When annexin A5 was added to the planar phospholipid surface, 2-D crystals of annexin A5 were observed, in dynamic imaging experiments, to grow from various nucleation sites until their meeting points (Figure 1A). As had been previously described,¹⁷ defects, having the appearance of furrows, were observed at the borders between the different crystals (Figure 1A). The entire phospholipid surface was covered by a layer of annexin A5 within 60 minutes (Figure 1, B and C). The vertical height of the annexin A5 2-D crystal layer formed over the phospholipid was determined to be $\sim 2.44 \pm 0.13$ nm. Five hundred measurements (100 measurements on five different experiments) were made on forming annexin A5 crystals by determining the vertical distance between the high peaks on the annexin A5 and the low valleys on a phospholipid furrow using the vertical section analysis feature of the Nanoscope software. In dynamic experiments, the addition of β_2 -GPI alone to the annexin A5 lattice had no observable effect on the lattice (Figure 1D). The addition of non-aPL control human monoclonal antibodies either after (Figure 1E) or before (Figure 1F) the addition of annexin A5, had no observable effect on the crystalline lattice structure.

Varying morphological effects were observed when the specific aPL mAbs and β_2 -GPI were added to the annexin A5 2-D crystal that had preformed on the planar phospholipid bilayer. The IS3 and CL15 mAbs produced the most consistent and dramatic effects on the annexin

Figure 1. Annexin A5 crystal structure. AFM images demonstrating the formation of an annexin A5 2-D crystal lattice on a planar phospholipid bilayer, and no effects of β_2 -GPI and a human control IgG antibody on the annexin A5 2-D crystal lattice. Amplitude image (A) shows irregular furrows (arrows) between annexin A5 lattices that are growing from different nucleation sites. Height images (B, C) show the completed 2-D crystalline lattice at different magnifications. The 100 nm $\times$ 100 nm zoomed height image (C) demonstrates the annexin A5 crystal lattice at a high resolution. In dynamic imaging experiments, the annexin A5 lattice is not affected by the addition of β_2 -GPI after 60 minutes of scanning (D); addition of control mAb IgG1 and β_2 -GPI to the annexin A5-covered bilayer also shows no effect on the crystal lattice (E). In an endpoint experiment, pretreatment of PS/PC bilayer with IgG1 and β_2 -GPI does not inhibit the formation of the annexin A5 2-D crystal lattice (F). Amplitude image (A) was processed with $\times 1$ convolution; the height image (C) was zero-flattened and low-pass filtered; the height images (B, E, F) were processed by zero order flatten mode.

A5 layer. The specific structural effect on the annexin A5 crystalline lattice varied, depending on the particular mAb. In dynamic imaging experiments, addition of β_2 -GPI alone had no discernible effect on the annexin A5 crystal lattice (Figure 2, A and B); however, circular deformities appeared after IS3 was added to the β_2 -GPI, indicating disruption of the crystal lattice (Figure 2, C and D).

The addition of CL15 alone had no effect (Figure 3A); however, the subsequent addition of β_2 -GPI resulted in a gross disruption of the annexin A5 crystal and the formation of large furrows and protein aggregates (Figure 3B). The addition of CL1 alone did not alter the structural integrity of the lattice, but a few globular structures appeared (Figure 3C). When CL1 was followed by β_2 -GPI, there was a subtle effect on the annexin A5 crystal struc-

Figure 2. Sequential effects of β_2 -GPI and IS3 on annexin A5 crystal structure. AFM images from a dynamic imaging experiment showing the effect of aPL mAb IS3 on a preformed annexin A5 crystalline lattice. The addition of β_2 -GPI alone has no discernible effect on the crystal lattice as observed using amplitude imaging (A) and at higher magnification with height imaging (B). On the subsequent addition of IS3, circular deformities appeared indicating disruption of the crystal lattice as seen in an amplitude image (C). A height image (D) within the circular deformities shows dark areas (white asterisk) representing portions of the surface that have lost annexin A5 coverage, near light areas (black asterisks) representing elevated structures, presumably antibody-antigen complexes. A and C: Amplitude images processed with a $\times 1$ convolution. B and D: Height images processed with a zero order flatten and low-pass filter.

ture, consisting of a loss of the recognizable lattice structure in some areas, with other areas remaining unaffected (Figure 3D).

The results of these dynamic experiments were corroborated with those from the endpoint-imaging experiments (Figures 4 and 5). When aPL mAb IS3 and β_2 -GPI were added to the annexin A5 crystal lattice preformed on the phospholipid bilayer, the crystal lattice was disrupted, as indicated by the appearance of circular pits (arrows in Figure 4, A and B, enlarged in insets). Moreover, at higher magnification (Figure 4, C and D), vacancy defects are readily observable in the 2-D annexin A5 crystal lattice. The addition of aPL mAb CL1 and β_2 -GPI to a previously formed annexin A5 crystal lattice in an endpoint experiment resulted in large aggregates forming on the crystal lattice (Figure 5, A and B). At higher magnification, it is apparent that the aggregates, presumably representing antibody-co-factor complexes, physically disrupted the annexin A5 crystal lattice (arrows in Figure 5C, arrows and arrowheads in 5D). In addition, the annexin A5 crystal lattice showed an abundance of vacancy defects.

We next observed the deposition of annexin A5 on phospholipid bilayers that had first been incubated with aPL mAbs and β_2 -GPI. In a series of endpoint-imaging experiments, the addition of IS3 (Figure 6, A and B), CL1 (Figure 6C), and CL15 (Figure 6D) together with β_2 -GPI to phospholipid, followed by the addition of annexin A5

Figure 3. Effects of other aPL mAbs on annexin A5 crystal structure. AFM images showing the effect of other aPL mAbs on previously formed annexin A5 2-D crystal in dynamic experiments. **A** and **C**: Annexin A5 surface in the presence of mAbs CL15 and CL1 before the addition of β_2 -GPI. After the addition of β_2 -GPI, CL15 grossly disrupts the annexin A5 crystal (**B**), forming large furrows (arrows), represented by dark areas. Note that in **C** the addition of mAb CL1 to the annexin A5 layer does not alter the structural integrity of the lattice in the absence of β_2 -GPI, but a few globular structures appear (arrows). **D**: On addition of co-factor β_2 -GPI, the globules enlarge (arrows) and the crystal structure is minimally disrupted (asterisk). Images (**B–D**) are height images with off-line zero-flatten and low-pass filtering applied; image in **A** is an amplitude image with no processing.

resulted in no visible annexin A5 crystal formation. Large irregularly shaped complexes with raised areas and disruptions of annexin A5 crystal lattice were observed (Figure 6).

Prothrombinase Activity Studies

Because the AFM images of the mAbs showed disruptions in the coverage of the phospholipid surfaces by annexin A5, we wanted to determine whether these antibodies might also affect the anticoagulant effect of annexin A5. In the absence of annexin A5, the addition of factor Xa, factor Va, and prothrombin to the phospholipid bilayer, resulted in a thrombin generation rate of 2110 pmol/L/minute. Annexin A5 (5 μ g/ml) markedly suppressed the thrombin generation rate to a level of 227 pmol/L/minute. The presence of β_2 -GPI promoted the reversal of the annexin A5 anticoagulant effect for all three of the mAbs and increased the thrombin generation rate to varying degrees. Two of the three aPL mAbs, CL1 and CL15, reversed the anticoagulant effect of annexin A5 to a lesser degree in the absence of β_2 -GPI, whereas one, IS3, had no inhibitory effect in the absence of the co-factor. The control IgG1 and IgG3 mAbs did not reverse annexin A5 anticoagulant activity whether or not β_2 -GPI was present. The results are shown in Table 1.

Figure 4. AFM images from endpoint-imaging experiments showing the effect of aPL mAb IS3 on a preformed annexin A5 crystal lattice. When IS3 and β_2 -GPI were added to the annexin A5 crystal lattice formed on the bilayer, circular pits appeared (arrows in **A** and **B**) indicating disruptions in the crystal lattice similar to those observed in the dynamic imaging experiments (Figure 2). A representative pit is shown at higher magnification in the insets. Moreover, at higher resolution (**C**, **D**) more vacancy defects (small round dark holes) in the crystalline lattice are apparent. Amplitude images (**A**, **C**) processed with a $\times 1$ convolution and height images (**B**, **D**) processed by zero order flatten. Original magnifications: 10 μ m $\times$ 10 μ m scan (**A**, **B**); 500 nm $\times$ 500 nm (**C**, **D**).

Discussion

Annexin A5 is a potent anticoagulant protein that serves a thrombomodulatory function in the placental circulation. The protein is highly expressed on the apical surfaces of placental syncytiotrophoblasts²¹ and has been demonstrated to be important for the maintenance of placental integrity,²⁷ protecting against fetal loss, placental thrombosis, and necrosis. Annexin A5 may also play a thrombomodulatory role in the systemic circulation. The protein is also highly expressed by human endothelial cells²⁸ and is present on the surfaces of endothelial cells.²⁹ Increased expression of annexin A5, via a Kozak polymorphism (-1C>T), is associated with a reduced risk of myocardial infarction in young men.³⁰

We previously demonstrated that aPL antibodies reduce the quantity of annexin A5 on the surfaces of cultured placental trophoblasts^{20,29,31} and vascular endothelial cells,²⁹ where they accelerate coagulation reactions and proposed that this may provide a mechanism for pregnancy losses and thrombosis in the aPL syndrome. We subsequently demonstrated by ellipsometry, with immunoassays and with labeled protein,¹³ that aPL antibodies reduce the quantity of annexin A5 on phospholipid surfaces and on platelets. These findings were confirmed by other investigators using phospholipid-coated microtiter plates¹⁴ and with platelets,¹⁵ but not by another group that used ellipsometry.¹⁶ We therefore used the imaging technique of AFM to visualize the ef-

Figure 5. AFM images showing the effect of aPL mAb CL1, together with β_2 -GPI on previously formed annexin A5 crystal in endpoint experiments. Amplitude images (A, C) are presented with corresponding height images (B, D). At a low magnification of $20\ \mu\text{m} \times 20\ \mu\text{m}$ scan (A, B), large aggregates (asterisks) presumably representing antibody/co-factor complexes are seen on the annexin A5 lattice. At higher magnification ($500\ \text{nm} \times 500\ \text{nm}$ scan) (C, D), displacement of the annexin A5 crystalline lattice at the site of CL1 + β_2 -GPI interaction is observed (arrowheads) with further disruption of the crystalline lattice indicated by an increased number of vacancy defects (arrows). The site of disruption is highlighted by the broken line in C. Images were minimally processed with off-line functions zero-flatten and erase scan line.

fects of aPL antibodies on the morphology of annexin A5 crystals.

This study is the first report of the application of AFM to investigate the pathophysiology of the aPL syndrome. Using annexin A5 alone, we confirmed that the protein formed 2-D crystals over phospholipid bilayers.¹⁰ Addition of the aPL mAbs, together with the major aPL co-factor β_2 -GPI, resulted in the formation of structures presumed to be antibody-antigen complexes on the phospholipid bilayer, together with evidence of disruption of the ordered crystallization of annexin A5 over the phospholipid surface. β_2 -GPI alone, β_2 -GPI together with the non-aPL control mAbs, and the aPL mAbs alone (ie, without β_2 -GPI) all had no observable effect on annexin A5 crystallization. Only the combination of each aPL mAb together with β_2 -GPI resulted in the formation of elevated structures and in visible disruptions of the ordered pattern of annexin A5 crystallization over the phospholipid bilayer. We speculate that the defects, or furrows, that occur between adjoining crystals that had originated from different nucleation sites (shown in Figure 1 and previously described by Reviakine et al¹⁰) may offer susceptible target areas for binding by aPL-antibody-co-factor complexes. We observed no significant reactivity, by AFM, between the mAbs and the annexin A5-covered surface in the absence of β_2 -GPI. It should be noted that although the human aPL mAbs were derived from immortalized lymphocytes of patients with the aPL syndrome, it

Figure 6. AFM images from endpoint experiments demonstrating effect of aPL mAb IS3, CL1, and CL15 on the subsequent formation of annexin A5 crystal. When annexin A5 is added to a planar phospholipid bilayer that had been preincubated with IS3 and β_2 -GPI, large aggregates formed over the phospholipid layer, but no annexin A5 crystal lattice is detected (A, B). aPL mAb CL1 shows a homogeneous disruptive effect on the forming annexin A5 crystal lattice (C), with large dark areas representing surface on which annexin A5 is not crystallized (arrows), whereas aPL mAb CL15 displays a grossly disruptive effect on the forming annexin A5 (D) leaving large dark patches uncovered by annexin A5. Amplitude image (A) was processed with a $\times 1$ convolution, height image (B) processed by zero order flatten, and height images (C, D) processed by zero-flatten, erase scan lines, and contrast enhancement.

is possible that the results of this study might not be generalizable to aPL antibodies that are present in serum.

It has become generally accepted that the major epitopes recognized by antiphospholipid antibodies are actually phospholipid-binding protein co-factors, primarily β_2 -GPI.³² β_2 -GPI is a member of the complement control protein or short consensus repeat (SCR) superfamily whose crystal structure suggests that the protein inserts directly into the phospholipid bilayer via the cationic portion of the fifth of its five SCR domains.^{33,34} It has been suggested that aPL antibody binding to other portions of the protein promotes its binding to membrane phospholipids,³³ perhaps as a consequence of the increased affinity of the divalent IgG- β_2 -GPI complexes.³⁵ The co-factor dependence for the disruption of the annexin A5 crystallization pattern that we observed with AFM is consistent with this concept. In a future study, we plan to investigate whether this disruption requires Fab₂ fragments of the mAbs.

We also studied the effects of these mAbs on annexin A5 anticoagulant activity. In accordance with previous reports that used IgG fractions from patients with the aPL syndrome,^{26,36} and in contrast to the report of another group,³⁷ we found that the aPL mAbs reversed the anticoagulant effect of annexin A5 and increased the generation of thrombin from the prothrombinase reaction. For one of the mAbs, IS3, the reversal of anticoagulant activity was completely dependent on β_2 -GPI, whereas for the other two mAbs, CL1 and CL15, partial reversal occurred

without the co-factor, but was augmented by its presence, suggesting that recognition of other epitopes may be involved in this process. As noted above in Materials and Methods, it is interesting that all three of these aPL mAbs weakly recognize cardiolipin directly, ie, in the absence of co-factor.¹⁹ This suggests that co-factor dependence may not be an absolute requirement for all aPL antibodies. It is also interesting that, in the absence of β_2 -GPI, the latter two mAbs increased thrombin generation but, by AFM, did not show a detectable effect on the crystallization of annexin A5. The reason for these differences remains to be determined and may be because of the methodological differences between the enzymatic and AFM systems, eg, the thrombin generation studies require stirring conditions, whereas the AFM studies must be performed under static conditions.

Although it has not yet been determined whether annexin A5 crystallization occurs on cell membranes *in vivo*, there is significant evidence to support such a possibility. For example, annexin A5 is abundantly expressed on the apical membranes of placental trophoblasts.²¹ Annexin A5 also binds to the surfaces of activated platelets³⁸ and to apoptotic cells and microparticles.³⁹ Also, measurable quantities of annexin A5 can be dissociated from the surfaces of cultured placental trophoblasts and human vascular endothelial cells.²⁹

As noted in Materials and Methods, the concentration of annexin A5 used for the imaging studies was based on practical considerations, because preliminary studies had shown that annexin A5 coverage of the phospholipid surface in the static, ie, nonstirring, conditions necessary for AFM imaging occurred throughout the course of ~1 hour at this protein concentration. Similarly, for the prothrombinase experiments, which are performed in a stirring system, the anticoagulant effect of annexin A5 was seen at a concentration of 5 μ g/ml. The level of annexin A5 in normal human serum is less than 10 ng/ml.²⁸ However, annexin A5 binding to sites such as the apical membranes of placental trophoblasts may occur from protein that is released locally by the cells, rather than from circulating blood, and the protein concentration within the adjacent microenvironment is not known. With respect to β_2 -GPI, the concentrations that we used, 15 to 30 μ g/ml, is in the range of concentrations that have been used in other studies with aPL antibodies and less than the lower end of the reference ranges, ie, 2 SD of mean, reported for the sera of normal men (73 μ g/ml) and women (88 μ g/ml).⁴⁰ The concentrations of the monoclonal antibodies that we used cannot be compared to the levels of polyclonal antibodies that are present in normal serum but were in a range that would constitute ~1% of the total serum IgG. We plan additional studies to evaluate the effects of varying the concentrations of reactants.

It should be noted that the prothrombotic mechanism described in this study is one of several that have been proposed for this syndrome.³ Among these, aPL antibodies have also been shown to increase the expression of adhesion molecules on endothelial cells,^{41,42} and to induce a proinflammatory and prothrombotic phenotype as a consequence of binding to β_2 -GPI on the endothelial surface.⁴³

In conclusion, we have described the first morphological evidence that aPL antibodies disrupt the formation of the annexin A5 anticoagulant shield. These results are consistent with previous findings, using other techniques,^{13–15} and contrast to other reports.^{16,37} The aPL antibody-mediated disruption of the anticoagulant annexin A5 lattice on phospholipid surfaces may be a pathophysiological mechanism for thrombosis and spontaneous pregnancy loss in the aPL syndrome. AFM offers a novel investigative tool for advancing the understanding of the pathophysiological role of aPL antibodies in this syndrome.

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