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Insights into the Pathophysiology of the Antiphospholipid Syndrome Provided by Atomic Force Microscopy

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Abstract

The antiphospholipid syndrome (APS) is an enigmatic autoimmune disorder in which patients present with thrombosis and/or recurrent pregnancy losses together with laboratory evidence for the presence of autoantibodies in the blood that recognize proteins that bind to anionic phospholipids – the most important of which is β_2 -glycoprotein I (β_2 GPI). Earlier, we hypothesized that the clinical manifestations arise from antibody-induced disruption of a 2-dimensional anticoagulant crystal shield, composed of annexin A5, present on placental trophoblast plasma membranes. Accordingly, we reasoned that a high resolution imaging technology such as atomic force microscopy could be used to investigate such molecular interactions at high resolution in a non-fixed hydrated environment. This review will focus on the contribution of this technique to the elucidation of the mechanism of APS.

Keywords

atomic force microscopy; antiphospholipid syndrome; annexin A5; β_2 glycoprotein I; hydroxychloroquine; thrombosis; placenta; recurrent pregnancy losses

1. Introduction

The antiphospholipid antibody (aPL Ab) syndrome (APS) is an autoimmune thrombotic disorder that was proposed to be a disease entity over 2 decades ago (Hughes et al., 1986). Despite the number of years that have elapsed since the initial description, the efforts that have been expended towards elucidating its molecular mechanism have yet to bear fruit. The autoantibodies that are generated by patients with the syndrome were initially thought to recognize phospholipids directly (specifically cardiolipin) (Hughes et al., 1986). However, the antibodies are now understood to bind to phospholipid-binding proteins, the most significant of which is β_2 -glycoprotein I (β_2 GPI) (Bever et al., 1991; Oosting et al., 1993, de Laat et al., 2004; De Laat et al., 2008; Rand et al., 2008b; 2010b). Despite this knowledge, the misnomers of “antiphospholipid” antibodies and syndrome have persisted. Studies of the syndrome have not evolved without some controversy involving the standard

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laboratory modalities employed to diagnose and assess the molecular phenomenon (Galli et al., 2003; de Laat et al., 2004; de Laat et al., 2008). This is exemplified by the absence of concordant clinical test results in the majority of patients who are diagnosed for the disorder. In addition, immunoassays (i.e., enzyme-linked immunosorbent assay (ELISA)) have displayed lack of specificity. Accordingly, de Laat and colleagues have called for a universal standardization of the ELISA assay (de Laat et al., 2004) with respect to β_2 GPI. Likewise, ellipsometry and coagulation tests (i.e., lupus anticoagulant (LA) tests) have provided much needed clinical information for APS, albeit accompanied by a lack of universal acceptance (Bever et al., 2000; Willems et al 2000). Investigating the antigenic determinants of β_2 GPI has resulted in the postulation of a variety of binding scenarios. A review by Giles et al (2003) reports one group (Matsuura et al., 1994) concluded that β_2 GPI must interact with lipid before an aPL mAb will bind to the complex. Mechanistically, they proposed that a conformational change to β_2 GPI upon binding to lipid must occur in order to expose an appropriate epitope(s) that the aPL mAb would subsequently recognize. Previously, Roubey et al. (1995) had proposed that it is not a cryptic epitope availability event but rather a density (forming patches/clusters) phenomenon, occurring on the lipid membranes. However, another group (Subang et al., 2000) reported opposing findings with no conformational change of β_2 GPI upon binding to phosphatidylserine; interestingly, they reported observing a conformational change upon binding to cardiolipin. Hamdan et al, (2007) corroborated that large-scale conformational changes of β_2 GPI occur upon binding to a target membrane. Their findings also support the premise that binding to lipid presents more than one conformational possibility (Bouma et al, 1999; Wang et al, 1999). More recently, convincing evidence has been presented that β_2 GPI circulates in plasma in a circular conformation and that the protein opens up when it binds to phospholipid bilayers (Agar et al., 2010). These authors provided evidence that the circular conformation results from affinity of a region in domain V near the carboxyterminus, containing the phospholipid binding site, for a region in domain I, near the aminoterminal. Binding of the protein to the phospholipid membrane via the domain V binding site requires the circular protein to snap open into a fishhook conformation. Given the contrasting and somewhat confusing nature of these biochemical results, we reasoned that high resolution visualization of these molecular interactions using fluid phase atomic force microscopy (AFM) could provide new insights on the molecular interactions underpinning APS and on novel approaches to treat the disorder. This review will focus on the novel findings relevant to the mechanisms of APS afforded by the use of high resolution AFM.

1.1 Advantages of Atomic Force Microscopy for Investigating Macromolecular Interactions

The atomic force microscope is a member of the scanning probe family of microscopes, which includes for instance the nearfield scanning optical microscope (NSOM; Betzig et al., 1986), the scanning tunneling microscope (STM; Binnig et al., 1982), and the magnetic force microscope (Han et al., 1986). The AFM microscope was first invented as a corollary to the STM; whereas the STM could measure surface forces occurring only on conducting samples, the AFM could measure forces on insulating materials as well (such as biological samples). In a sense, the AFM was conceived as a combination STM/surface profilometer (Binnig et al., 1986) for measuring “atomic forces” occurring at the surface between a fine tip and an insulating (or conducting) surface. However, probe-sample interactions can also be converted into a topological surface map of the sample. In its simplest form, AFM “features a tactile sensing system, in which a silicon or silicon nitride probe is attached to the end of a flexible cantilever and rasterizes in an x and y direction across a specimen’s topography through the activity of a piezoelectric tube to which the cantilever assembly is mounted” (Montigny et al., 2006). Although three principle AFM imaging modes exist (contact, non-contact, and tapping (AC mode)), the majority of biomolecule imaging utilizes AC mode. In this mode, as the cantilever oscillates at its resonance frequency, contact with

the sample surface decreases the oscillation amplitude. This amplitude effect is detected by an infrared laser which is reflected off of the back of the highly reflective cantilever and detected by a photodiode. The force between the cantilever tip and the sample surface is kept constant by a feedback loop mechanism, and as the tip raster scans across the sample surface, a topographic map is generated by the feedback output (Katan and Dekker, 2011). The immense advantage of AFM over other microscopy-based imaging modalities is that resolution on the order of tens of nanometers can be realized on unfixed, hydrated soft biological samples. Sample preparation is minimal, with specimens typically adhered to an atomically flat muscovite mica disk. We will review how this tactile imaging technique visually presents temporal conformational changes of a selective plasma protein and aPL mAbs interacting on an artificial PSPC model planar lipid layer in a physiological environment. Dynamic “Tapping Mode” imaging with continuous capture allowed our laboratory to record molecular reactions of the aPL mAbs with an antigenic catalyst - plasma protein β_2 GPI (Rand et al., 2003; Rand et al., 2008a). Our AFM studies have included observing the temporal and spatial interactions of AnxA5, β_2 GPI, aPL mAbs, and control IgGs with planar lipid layers and each other on a mica support matrix as an *in vitro* simulacrum. Moreover, this system allows the imaging of the effects of candidate drugs that might interfere with the aPL-mediated disease process. To this end, we have utilized AFM to report on the effects of the immunomodulatory drug hydroxychloroquine (HCQ) on formed aPL mAb- β_2 GPI complexes and the AnxA5 two dimensional crystal shield (Rand et al., 2008a; 2010a).

A major advantage of AFM for approaching this research question is that one can continually assess the effects of various sequential combinations of plasma proteins interacting together and with planar lipid layers in specific physiological buffers with an *in situ* molecular concentration (1.25mmol/L) of calcium chloride (CaCl_2); for example, as reported in earlier mechanistic studies of AnxA5-membrane interactions with other modalities (Tait & Gibson, 1992). Our novel results with AFM were corroborated by ellipsometry (Rand et al., 1998) and other analytical modalities such as coagulation enzyme reactions (Rand et al, 2003), immunoassays (Rand et al, 1998), competitive binding assays with fluorescent-labeled ligands (Rand et al, 2004; Rand et al, 2006), biochemistry (Rand et al., 2003; Rand et al., 2008a), and confocal microscopy of the localization of the proteins on cultured cells (Wu et al, 2011).

2. Atomic Force Microscopy to Investigate APS

Our AFM studies were performed using a Veeco BioScope (with a Nanoscope IIIa controller) attached to an Olympus IX 70 microscope stand via a specially designed rigid stage. Samples were imaged in fluid with continuous capture engaged, using proprietary Veeco “Tapping Mode” with pyramidal 100 μm length oxide-sharpened silicon nitride probes (Taates et al., 1999; Rand et al., 2003; Montigny et al., 2006). Tapping mode AFM is the imaging mode of choice for soft biological specimens. As described earlier, in this AFM mode, the scanning probe makes intermittent contact with the sample, thus minimizing potentially damaging interactions prevalent with “Contact Mode” imaging. Temporal images were acquired every 2–10 minutes, with the scan rate and scan size determined by the nature of the sample and the desired information to be collected. Images were acquired with both height and amplitude information: the height information allows for accurate quantitative topographical analysis, while the amplitude information provides an “error correction signal” which essentially provides sharp qualitative images (Montigny et al., 2006). Calcium chloride concentrations present in the imaging buffers were adjusted as necessary dependent upon the molecular reactions to be observed.

It is well established that an atomically flat surface is ideal for absorbing and fusing lipid vesicles into a homogenous planar membrane conducive for the observation of molecular interactions between proteins and lipids. Benes and colleagues (2002; 2004) validated the use of muscovite mica with miniscule surface roughness as a base platform that would provide reproducible planar flatness for nanoscale AFM imaging.

The in vitro simulacrum that we applied to investigate molecular mechanisms involved in APS included a planar lipid layer composed of a phospholipid vesicle mixture of 30% phosphatidylserine and 70% phosphatidylcholine (PSPC) formed on freshly-cleaved muscovite mica (Rand et al., 2003), employing the fusion method (Brian and McConnell, 1984; McConnell et al., 1986; Reviakine and Brisson, 2000). After the initial incubation and rinse steps, precautions were followed to prevent oxidation of the forming planar phospholipid model membrane to maintain a homogenous surface (absent defects and voids) via repeated aspiration and rinsing technique with the same buffer. As visualized by fluid tapping-mode AFM, these planar lipid layers formed a 4.0 nm thick homogeneous substrate on the mica surface. Next, the lipid substrates were either used immediately for incubation with β 2GPI and/or other proteins to maximize formation(s) and/or reaction(s) as described below, or stored at 4°C until needed. A subsequent rinse with the physiological buffer was always performed before use in an AFM imaging session.

Our earlier imaging data from lipid model membranes and AnxA5 formation consisted of images displaying AnxA5 transitionally forming with voids (defects) created by the presence of preabsorbed components such as BSA (Rand et al., 2003; Montigny et al., 2006) or atmospheric exposure during a gross rinsing procedure. We have shown the effect of BSA on lipid bilayer formation (Montigny et al., 2006) and, here, present improved image clarity via electronically zoomed images (Fig. 1). Willems and co-workers (Bever et al., 2000; Willems et al., 2000) posited that the inclusion of BSA in the lipid reaction media used for coating silicon substrates for ellipsometry prevented the formation of a continuous lipid surface. Indeed, our early experimental AFM data indicated that BSA pre-bound to the lipid membrane resulted in incomplete and heterogeneous adsorption and 2-dimensional crystallization of the protein, AnxA5. The AnxA5 crystal was observed to form around the defects in the planar lipid layer, which were ostensibly caused by the inclusion of BSA in the buffer medium. Moreover, it is possible that the exposure of the lipid surface to air could potentially compromise the accuracy of image interpretation when other components are added before and after AnxA5 addition. Although the presence of such voids did concurrently enable height measurements of the encompassing AnxA5 2-D crystal formation, a complete homogenous lipid surface was desired as an optimal surface substrate for harboring subsequent molecular interactions. Therefore, all our AFM experimental (published) data to date were obtained without the use of BSA in the lipid-forming buffer. Using the lipid fusion method and our subsequent rinsing procedure as described above, consistently provided intact menisci with no run-off from the atomically flat mica substrate.

The proteins that we utilized were chosen to mimic the disease process within the capacities of the imaging system. Since initial pilot studies indicated effective concentrations of polyclonal IgG aPL antibodies derived from patient sera prevented the ability to image phospholipid bilayers because the viscosity of the resulting buffer interfered with the ability of the probe to resolve differences in height, we decided to proceed with monoclonal aPL antibodies that were generated from the peripheral blood mononuclear cells from APS patients as previously described (Zhu et al., 1999; Lin et al., 2007). By ELISA, the aPL antibodies designated IS3 and IS4 reacted strongly against human cardiolipin- β 2GPI complexes; however, they displayed weak reactivity against cardiolipin alone. As a result of their determined level of reactivity, we chose to investigate the effect of these two aPL

mAbs on AnxA5 binding to PSPC lipid model membrane via AFM (Rand et al., 2003; Montigny et al. 2006; Rand et al., 2008a).

AnxA5, previously known by several other names, including annexin-V, placental anticoagulant protein-1, and vascular anticoagulant- α , was purified from human placentas according to the methods of Funakoshi and colleagues (Funakoshi et al., 1987; Tait et al., 1988). AnxA5, also used as a popular cytochemical marker for apoptotic cells in culture (Taatjes et al., 2008a), has been shown to be expressed at the cell surface of placental trophoblasts and endothelial cells, where it crystallizes over anionic phospholipids serving as an anticoagulant protective shield (Rand et al., 1994). The potent anticoagulant properties of AnxA5 have been attributed to its forming two-dimensional (2-D) crystals over phospholipid surfaces (Mosser et al., 1991; Tait & Gibson, 1992; Olofsson et al., 1994; Voges et al., 1994; Reviakine et al., 1998; Reviakine et al., 2000; Han et al., 2008; Van Genderen et al., 2008; Irman et al., 2009) that shield the underlying phospholipid moieties from availability for phospholipid-dependent coagulation reactions (Andree et al., 1992).

Human β_2 GPI, also known as apolipoprotein H, is a highly glycosylated protein, consisting of 326 aminoacids that was discovered in 1990 to be a cofactor for anti-cardiolipin antibodies from patients with APS (McNeil et al., 1989; Galli et al., 1990; Matsuura et al., 1992). β_2 GPI appears to correlate strongly with a significantly increased risk of thrombosis (Galli et al., 2003; de Laat et al., 2004).

We analyzed the adsorptions of these proteins to planar phospholipid bilayers with AFM. Initially, at the beginning of imaging sessions we acquired amplitude force plot(s) (Fig. 2) to verify the presence of a soft surface, indicating the formation of a planar lipid layer over the cleaved mica substrate. Likewise, in conjunction with developing a consistent planar lipid preparation, we established a reliable protocol for creating a well-defined AnxA5 2D crystal on the planar lipid bilayer (Montigny et al., 2006; Rand et al., 2008b). As previously published, we determined a final AnxA5 concentration range of 40 $\mu\text{g/ml}$ – 48 $\mu\text{g/ml}$, and concomitantly established that it requires approximately 45–60 minutes at room temperature for AnxA5 crystal formation to ensue (Fig. 3); completion of the 2-D crystal covering the entire lipid surface required a further 60 minutes or more when using the physiological concentration of CaCl_2 (1.25 mmol/L). The time required for AnxA5 2-D crystal formation could be lessened by increasing the concentration of CaCl_2 added to the reaction buffer (up to 2 mmol/L). Other groups have also used AFM to image AnxA5 crystal formation on planar lipid bilayers. Indeed, the pioneering work of Brisson and co-workers (Reviakine et al., 1998; 2000; Oling et al., 2001; Richter et al., 2005) utilized AnxA5 as a model protein for investigating the formation of a 2-D crystal on a supported planar lipid bilayer. They were the first group to not only demonstrate by AFM the 2-D structure of AnxA5 crystals formed on a planar lipid layer, but also to identify structural defects in the crystal (Reviakine et al., 1998), the topography of the p3 and p6 crystal forms of AnxA5 (Reviakine et al., 2000), as well as a detailed analysis of the kinetics and environmental characteristics of AnxA5 crystal formation (Richter et al., 2005). More recently, Irman and coworkers used AFM to confirm that, in similarity to their assembly on phospholipid bilayers composed of purified phosphatidylserine and phosphatidylcholine, the crystal forms of AnxA5 also assemble on a variety of naturally-derived phospholipid surfaces (Irman et al., 2009).

We also performed AFM imaging experiments after the reactions had reached equilibrium (termed “static” or “endpoint” experiments in contrast to “dynamic” imaging experiments) to eliminate the possibility that the mechanics of the AFM silicon nitride probe, while physically tapping on the surface of the lipid membrane and/or the proteins themselves could alter or enhance the molecular interactions occurring during the dynamic experiments (Rand et al., 2003). After the necessary protocols were established for creating

homogeneous lipid bilayers on cleaved mica disks, followed by forming AnxA5 2-D crystals on top of this lipid layer, we used AFM to investigate the molecular effect of the addition of β_2 GPI and patient-derived aPL antibodies to the established AnxA5 crystal (Rand et al., 2003).

The cofactor protein, β_2 GPI, was slow to form binding sites on the model lipid membrane (fused on a 1.2 cm freshly-cleaved mica disc surface) when employing a physiological concentration of 1.25 mmol/L CaCl_2 in the buffer. When adding the β_2 GPI dynamically, a higher concentration was required via subsequent bolus additions to achieve a more immediate binding and oligomerized formation (Roubey et al., 1995; Gamsjaeger et al., 2005). The subsequent reactivity of β_2 GPI could be a result of its ability to form higher molecular volume agglomerates to patch-like structures (clusters), providing a larger number of conformationally exposed and available binding epitopes. More recent experiments, requiring increased time to image and record, warranted pre-incubating the β_2 GPI on the planar lipid layer at 0.7 $\mu\text{g/ml}$ overnight at 4°C. Using this protocol, we obtained completely formed and stable patch-like sites of β_2 GPI cofactor agglomerations, to which AnxA5 and aPL antibodies could be subsequently added. With these overnight incubations, we obtained similar timed-resolved binding of β_2 GPI onto PSPC planar lipid layers (Figs. 5–9) as reported by Gamsjaeger and colleagues (2005). The agglomeration of β_2 GPI on the planar lipid layers was observed to occur in a two-step mechanism: (1) initial binding of the cofactor to the lipid (see Figure 15.3 A–B in Montigny et al., 2006); and (2) subsequent formation of clusters of variable sizes (Figs. 4–9); see also Figure 5 A–B in Rand et al., 2003). Subsequently added aPL antibodies were found to bind to the β_2 GPI clusters, enhancing their size and complexity (Figs. 5–6), as proposed by Gamsjaeger and co-workers (2005). Interestingly, it has been hypothesized that increased agglomerations of β_2 GPI in plasma and on lipid surfaces (Frank et al., 2009) present a very conducive antigenic target site for aPL antibodies to bind. Moreover, with such suspected epitope specificity, the aPL mAb- β_2 GPI complexes will likely develop into conformationally enhanced structures capable of interfering with, or disrupting, the anticoagulant protective AnxA5 crystalline shield of the placental membrane adjacent to the maternal blood. Current investigations in our laboratory are addressing in greater detail the binding between β_2 GPI complexes and aPL antibodies, including using recombinant β_2 GPI as a primary reagent.

The addition of β_2 GPI and specific monoclonal aPL antibodies derived from patients with primary APS to a previously formed AnxA5 2-D crystal surface on a planar lipid layer, resulted in disruption of the 2-D crystal (Figs. 3, 5, 6) (Rand et al., 2003); the degree of disruption, as assessed by AFM was antibody-dependent. Resultant defects ranged from minor “vacancies” to gross disruption of the 2-D crystal. Similar results were observed with both “dynamic imaging” and “endpoint imaging”. In dynamic imaging experiments, the preparations were imaged continuously in buffer as the components were added in a sequential fashion. Endpoint (static) imaging experiments were performed as single observations after all of the components had been added and a pre-determined time had elapsed. Importantly, AnxA5 disruption by the aPL antibodies was only manifested in the presence of β_2 GPI; in its absence, the aPL antibodies had no visible effect on the 2-D AnxA5 crystal structure (see Rand et al., 2003). Accordingly, this was the first demonstration by *in vitro* AFM that patient aPL monoclonal antibodies, in tandem with β_2 GPI, could physically disrupt the anticoagulant AnxA5 shield on planar lipid surfaces, offering tangible visible evidence for the etiology of APS as proposed by Rand (Rand, 2002). These results have recently been corroborated by Irman and colleagues (2011).

It should be noted that studies performed by Poindron and colleagues with another monoclonal aPL IgG did not show disruption of AnxA5 crystallization (Poindron et al., 2011); this is not unexpected since aPL antibodies are known to be heterogeneous and have

reactivities to a wide number of epitopes (Lieby et al., 2001). In accordance with this idea, our own clinical studies have indicated that while a substantial proportion of patients with APS have evidence for antibodies that disrupt AnxA5 anticoagulant activity, a subset of patients do not show evidence for this disruption (Rand et al, 2004; Hunt et al, 2011), suggesting that other aPL-mediated disease mechanisms may also be operative in individual patients.

3. AFM Assessment of the Modulation of APS by Pharmacological Intervention

The above observations led to the intriguing question of whether putative candidate therapies for this aPL-mediated disease process might be detected through this imaging technology. This section will address our recent AFM efforts directed at molecular interventions to repair the defects in the AnxA5 2-D crystal anticoagulant shield initiated by exposure to β_2 GPI and aPL monoclonal antibodies.

For these studies, we utilized the simulacrum described above to which we added candidate molecules. The synthetic antimalarial drug, hydroxychloroquine (HCQ) was a particularly attractive candidate because retrospective clinical studies have indicated that there is a reduction in the frequency of thrombosis among patients who take the drug (Petri, 1996; Erkan et al., 2002). However, it should be noted that prospective clinical trials have not yet been done and are currently being planned by the international collaborative network APS ACTION <http://www.apsaction.org>.

HCQ is an amphiphilic antimalarial molecular compound that is widely used for nonsteroidal immunosuppressive treatment of systemic lupus erythematosus (SLE), for SLE during pregnancy (Rothfield, 1988; Nayak & Esdaile, 1996; Tsakonas et al., 1998; Molad et al., 2002; Fessler et al., 2005; Clowse et al., 2006; James et al., 2007) and for rheumatoid arthritis. Since the recognition of β_2 GPI by anti-aPL antibodies plays a central role in the aPL syndrome, we investigated by AFM whether the reported reduction of thrombosis might be attributable to a HCQ-mediated impact on the formation of β_2 GPI-aPL antibody complexes on planar lipid layers. HCQ was prepared as described in HEPES-buffered saline (HBS) to yield final concentrations of 1 mg/ml or 1 μ g/ml. In a typical experiment, β_2 GPI was added to a planar lipid layer (PSPC), and incubated overnight at 4°C. The remainder of the experiment including the varying orders of addition of the proteins and the drug was performed the following day, as previously described (Rand et al., 2008a). Quantitative evaluation of 40 β_2 GPI-anti-aPL antibody complexes revealed that the complex height was reduced from a mean value of 17.7 ($\pm$ 5.8) nm to 9.3 ($\pm$ 2.4) nm upon addition of HCQ (Rand et al., 2008a). These AFM direct imaging results showing the effect of HCQ on β_2 GPI-anti-aPL antibody complexes (Fig. 8) were corroborated by ellipsometry (Rand et al., 2008a; see Figure 1C), the binding of aPL antibodies to THP-1 monocytes (Rand et al., 2008a; see Figure 3), and several clinical assays used for APS diagnosis (Rand et al., 2008a).

Based upon these encouraging results, we next determined whether the AnxA5 anticoagulant shield disruption promoted by exposure to β_2 GPI-anti-aPL antibody complexes might be reversed by addition of HCQ. Indeed, in the presence of HCQ, a second layer of AnxA5 crystals formed around and over the β_2 GPI-anti-aPL antibody complexes, in essence producing a “patch” of the damaged planar lipid layer induced by binding of the β_2 GPI cofactorantibody complex (Fig. 9; Rand et al. 2010a).

Our current efforts are directed towards unraveling the molecular mechanisms responsible for the “patching” effect HCQ manifests towards the damaged AnxA5 crystal formed on

artificial lipid bilayers. We are also investigating the molecular interactions between HCQ and the aPL immune complexes with chemical modifications of HCQ and with recombinant forms of the proteins.

4. Potential Limitations of AFM Imaging

AFM imaging has proven to be very useful for investigating APS. In addition to confirming and validating concepts that had been developed from immunohistochemical observations and from quantitative binding studies, this technique has yielded novel observations. Examples of findings that would have been missed with the other methods include the observations that furrows that represent defects in crystallization are “attack” sites for the binding of aPL- β 2GPI, that observations that binding of immune complexes disrupts the crystallization of AnxA5, and the finding that the antimalarial drug HCQ promotes a second layer of AnxA5 crystal over areas of the membrane where crystallization was disrupted by the aPL- β 2GPI immune complexes.

However, all simulacrum/simulacra/models have inherent limitations and can only provide an approximation of the biologic milieu. Below, we will cite a few examples of how conditions used for AFM experimental models of the APS disease process may differ from *in vivo* reality. For example, experiments cannot be done using a serum or plasma milieu because the viscosity would interfere with the ability of the cantilever to provide accurate imaging data. Solutions also that contain concentrations of IgG equivalent to those present in serum also have a viscosity that make it difficult to generate AFM images. For that reason, our experiments have utilized monoclonal aPL and control antibodies. The last example we will mention involves the concentrations of phospholipids. Although it is known that phosphatidyl serine becomes expressed on the extracellular leaflets of cytoplasmic membranes during the progression of the apoptotic cascade, the exact concentration of that phospholipid is not known. It is possible that the concentrations used for making planar bilayers in our experiments are different from the concentrations present in living cells.

5. Conclusions

The molecular basis for thrombosis in APS has been resistant to elucidation. AFM has proven to be a very useful tool for investigating a hypothesized aPL-mediated pathogenic mechanism because this high resolution analytical imaging instrumentation enabled us to investigate these interactions at the molecular level in a hydrated, unfixed environment. AFM provided the first visual evidence supporting the premise that aPL antibodies, in tandem with β 2GPI, could disrupt the anticoagulant AnxA5 two dimensional crystal shield on a planar lipid layer. Thus, this instrumentation, originally developed as a means to measure forces interacting at sample surfaces, provided the high resolution imaging means necessary to uncover the molecular events underlying a clinical conundrum and to validate the mechanism of novel targeted treatments, such as HCQ.

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References

- Agar C, van Os GM, Mörgelin M, Sprenger RR, Marquart JA, Urbanus RT, Derksen RH, Meijers JC, de Groot PG. Beta2-glycoprotein I can exist in 2 conformations: implications for our understanding of the antiphospholipid syndrome. *Blood*. 2010; 26:1336–1343. [PubMed: 20462962]
- Andree HAM, Stuart MCA, Hermens WT, Reutelingsperger CPM, Hemker HC, Frederik PM, Willems GM. Clustering of lipid-bound annexin V may explain its anticoagulant effect. *J. Biol. Chem.* 1992; 267:17907–17912. [PubMed: 1387643]
- Benes M, Billy D, Benda A, Speijer H, Hof M, Hermens WT. Surface-dependent transitions during self-assembly of phospholipid membranes on mica, silica and glass. *Langmuir*. 2004; 20:10129–10137. [PubMed: 15518504]
- Benes M, Billy D, Hermens WT, Hof M. Muscovite (mica) allows the characterization of supported bilayers by ellipsometry and confocal fluorescence correlation spectroscopy. *Biol. Chem.* 2002; 38:337–341. [PubMed: 11934273]
- Betzig E, Lewis A, Harootunian A, Isaacson M, Kratschmer E. Near-field scanning optical microscopy (NSOM): Development and biophysical applications. *Biophys. J.* 1986; 49:269–279. [PubMed: 19431633]
- Bevens EM, Galli M, Barbui T, Comfurius P, Zwaal RFA. Lupus anticoagulant IgG's (LA) are not directed to phospholipids only, but to a complex of lipid-bound human prothrombin. *Thromb. Haemost.* 1991; 66:629–632. [PubMed: 1796407]
- Bevens EM, Janssen MP, Willems GM, Zwaal FA. No evidence for enhanced thrombin formation through displacement of annexin V by antiphospholipid antibodies. *Thromb. Haemost.* 2000; 83:792–794. [PubMed: 10823284]
- Binnig G, Quate CF, Gerber C. Atomic force microscope. *Phys. Rev. Lett.* 1986; 56:930–933. [PubMed: 10033323]
- Binnig G, Rohrer H, Gerber C, Weibel E. Surface studies by scanning tunneling microscopy. *Phys. Rev. Lett.* 1982; 49:57–61.
- Bouma B, de Groot PG, van den Elsen JH, Ravelli RG, Schouten A, Simmelink MA, Derken RM, Kroon J, Gros P. Adhesion mechanism of human β_2 -glycoprotein I to phospholipids based on its crystal structure. *EMBO J.* 1999; 18:5166–5174. [PubMed: 10508150]
- Brian AA, McConnell HM. Allogeneic stimulation of cytotoxic T cells by supported planar membranes. *Proc. Natl. Acad. Sci. USA.* 1984; 81:6159–6163. [PubMed: 6333027]
- Clowse MEB, Magder L, Witter F, Petri M. Hydroxychloroquine in lupus pregnancy. *Arthritis & Rheumatism*. 2006; 54:3640–3647. [PubMed: 17075810]
- de Laat HB, Derksen RHW, de Groot PG. β_2 -glycoprotein I, playmaker in the antiphospholipid syndrome. *Clin. Immunol.* 2004; 112:161–168. [PubMed: 15240159]
- de Laat HB, Mertens K, de Groot PG. Mechanisms of disease: antiphospholipid antibodies-from clinical association to pathologic mechanism. *Nat. Clin. Pract. Rheumatol.* 2008; 4:192–199. [PubMed: 18285765]
- Erkan D, Yazici Y, Peterson MG, Sammaritano L, Lockshin MD. A cross-sectional study of clinical thrombotic risk factors and preventive treatments in antiphospholipid syndrome. *Rheumatology (Oxford)*. 2002; 41:924–929. [PubMed: 12154210]
- Fessler BJ, Alarcon GS, McGwin G Jr, Roseman J, Bastian HM, Friedman AW, Baethge BA, Vila L, Reveille JD. Systemic lupus erythematosus in three ethnic groups: XVI association of hydroxychloroquine use with reduced risk of damage accrual. *Arthritis & Rheumatism*. 2005; 52:1473–1480. [PubMed: 15880829]
- Frank M, Sodin-Semri S, Irman S, Bozic B, Rozman B. β_2 -glycoprotein I and annexin A5 phospholipid interactions: Artificial and cell membranes. *Autoimmun. Rev.* 2009; 9:5–10. [PubMed: 19232551]
- Funakoski T, Heimark RL, Hendrickson LE, Mc Mullen BA, Fujikawa K. Human placental anticoagulant protein: isolation and characterization. *Biochemistry*. 1987; 26:5572–5578. [PubMed: 2960376]

- Galli M, Comfurius P, Maassen C, Hemker HC, debates MH, van Breda-Vriesman PJC, Barbui T, Zwaal RFA, Bevers EM. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor. *Lancet*. 1990; 335:1544–1547. [PubMed: 1972485]
- Galli M, Luciani D, Bertolini G, Barbui T. Lupus anticoagulants are stronger risk factors for thrombosis than anticardiolipin antibodies in the antiphospholipid syndrome: a systematic review of the literature. *Blood*. 2003; 101:1827–1832. [PubMed: 12393574]
- Gamsjaeger R, Johs A, Gries A, Gruber HJ, Romanin C, Prassl R, Hinterdorfer P. Membrane binding of β_2 -glycoprotein I can be described by a two-state reaction model: an atomic force microscopy and surface plasmon resonance study. *Biochem. J*. 2005; 389:665–673. [PubMed: 15813706]
- Giles I, Isenberg DA, Latchman DS, Rahman A. How do antiphospholipid antibodies bind β_2 -glycoprotein I? *Arthritis & Rheumatism*. 2003; 48:2111–2121. [PubMed: 12905463]
- Hamdan R, Maiti SN, Schroit AJ. Interaction of β_2 -glycoprotein I with phosphatidylserine-containing membranes: ligand-dependent conformational alterations initiate bivalent binding. *Biochemistry*. 2007; 46:10612–10620. [PubMed: 17715943]
- Han JJ, Park S-H, Boo DW. A correlated force-optical study on the self-assembly behavior of annexin V on model membranes: effect of dye conjugation. *J. Phys. Chem. B*. 2008; 112:12175–12178. [PubMed: 18783196]
- Han W, Lindsay SM, Jing T. A magnetically driven oscillating probe microscope for operation in liquids. *Appl. Phys. Lett.* 1996; 69:1–3.
- Hughes GR, Harris NN, Gharavi AE. The anticardiolipin syndrome. *J. Rheumatol.* 1986; 13:486–489. [PubMed: 3735270]
- Hunt BJ, Wu XX, de Laat B, Arslan AA, Stuart-Smith S, Rand JH. Resistance to annexin A5 anticoagulant activity in women with histories for obstetric antiphospholipid syndrome. *Am. J. Obstet. Gynecol.* 2011; 205:485.e17–485.e23. [PubMed: 21784397]
- Irman S, Skarabot M, Musevic I, Rozman B, Bozic B. The use of atomic force microscopy to study the pathologic effects of anti-annexin autoantibodies. *J. Autoimmun.* 2011; 36:95–108.
- Irman S, Skarabot M, Igor M, Rozman B, Bozic B. *In vitro* model of annexin A5 crystallization on natural phospholipid bilayers observed by atomic force microscopy. *Autoimmunity*. 2009; 42:414–442. [PubMed: 19811258]
- Katan AJ, Dekker C. High-speed AFM reveals the dynamics of single biomolecules at the nanometer scale. *Cell*. 2011; 147:979–982. [PubMed: 22118456]
- James JA, Kim-Howard XR, Bruner BF, Jonsson MK, McClain MT, Arbuckle MR, Walker C, Dennis GJ, Merrill JT, Harley JB. Hydroxychloroquine sulfate treatment is associated with later onset of systemic lupus erythematosus. *Lupus*. 2007; 16:401–409. [PubMed: 17664230]
- Lieby P, Soley A, Levallois H, Hugel B, Freyssinet JM, Cerutti M, Pasquali JL, Martin T. The clonal analysis of anticardiolipin antibodies in a single patient with primary antiphospholipid syndrome reveals an extreme antibody heterogeneity. *Blood*. 2001; 97:3820–3828. [PubMed: 11389022]
- Lin W-S, Chen P-C, Yang C-D, Cho E, Hahn BH, Grossman J, Hwang K-K, Chen PP. Some antiphospholipid antibodies recognize conformational epitopes shared by β_2 -glycoprotein I and the homologous catalytic domains of several serine proteases. *Arthritis Rheum.* 2007; 56:1638–1647. [PubMed: 17469158]
- Matsuura E, Igarashi Y, Fujimoto M, Ichikawa K, Suzuki T, Sumida T, Yasuda T, Koike T. Heterogeneity of anticardiolipin antibodies defined by the anticardiolipin cofactor. *J. Immunol.* 1992; 148:3885–3891. [PubMed: 1602135]
- Matsuura E, Igarashi Y, Yasuda T, Triplett DA, Koike T. Anticardiolipin antibodies recognize β_2 -glycoprotein I structure altered by interacting with an oxygen modified solid phase surface. *J. Exp. Med.* 1994; 179:457–462. [PubMed: 7507506]
- McConnell HM, Watts TH, Weis RM, Brian AA. Supported planar membranes in studies of cell-cell recognition. *Biochim. Biophys. Acta*. 1986; 864:95–106. [PubMed: 2941079]
- McNeil HP, Chesterman CN, Krilis SA. Anticardiolipin antibodies and lupus anticoagulants comprise separate antibody subgroups with different phospholipid binding characteristics. *Br. J. Haematol.* 1989; 73:506–513. [PubMed: 2514785]

- Molad Y, Gorshtein A, Wysenbeek AJ, Guedj D, Majadla R, Weinberger A, Amit-Vazina M. Protective effect of hydroxychloroquine in systemic lupus erythematosus. Propective long-term study of an Israeli cohort. *Lupus*. 2002; 11:356–361. [PubMed: 12139373]
- Montigny, WJ.; Quinn, AS.; Wu, X-X.; Bovill, EG.; Rand, JH.; Taatjes, DJ. Atomic force microscopy in the study of macromolecular interactions in hemostasis and thrombosis: utility for investigation of the antiphospholipid syndrome. In: Jena, BJ.; Horber, JKH., editors. *Force microscopy: applications in biology and medicine*. Hoboken: Wiley; 2006. p. 267-286.
- Mosser G, Ravanat C, Freyssinet J-M, Brisson A. Sub-domain structure of lipid-bound annexin-V resolved by electron image analysis. *J. Mol. Biol.* 1991; 217:241–245. [PubMed: 1825119]
- Nayak V, Esdaile JM. The efficacy of antimalarials in systemic lupus erythematosus. *Lupus*. 1996; 5(Suppl 1):S23–S27. [PubMed: 8803906]
- Oling F, Bergsma-Schutter W, Brisson A. Trimers, dimmers of trimers, and trimers of trimers are common building blocks of annexin A5 two-dimensional crystals. *J. Struct. Biol.* 2001; 133:55–63. [PubMed: 11356064]
- Olofsson A, Mallouh V, Brisson A. Two-dimensional structure of membrane-bound annexin V at 8 Å resolution. *J. Struct. Biol.* 1994; 113:199–205. [PubMed: 7734244]
- Oosting JD, Derken RHWM, Bobbink IWG, Hackeng TM, DeGroot PG. Antiphospholipid antibodies directed against a combination of phospholipids with prothrombin, protein C, or S: an explanation for their pathogenic mechanism? *Blood*. 1993; 81:2618–2625. [PubMed: 8166780]
- Petri M. Thrombosis and systemic lupus erythematosus: the Hopkins Lupus Cohort perspective. *Scand. J. Rheumatol.* 1996; 25:191–193. [PubMed: 8792794]
- Poindron V, Berat R, Knapp AM, Toti F, Zobairi F, Korganow AS, Chenard MP, Gounou C, Pasquali JL, Brisson A, Martin T. Evidence for heterogeneity of the obstetric antiphospholipid syndrome: thrombosis can be critical for antiphospholipid-induced pregnancy loss. *J. Thromb. Haemost.* 2011; 9:1937–1947. [PubMed: 21848640]
- Rand JH. Molecular pathogenesis of the antiphospholipid syndrome. *Circ. Res.* 2002; 90:29–37. 1566–1572. [PubMed: 11786515]
- Rand JH, Arslan AA, Wu XX, Wein R, Mulholland J, Shah M, van Heerde WL, Reutelingsperger CP, Lockwood CJ, Kuczyński E. Reduction of circulating annexin A5 levels and resistance to annexin A5 anticoagulant activity in women with recurrent spontaneous pregnancy losses. *Am. J. Obstet. Gynecol.* 2006; 194:182–188. [PubMed: 16389029]
- Rand JH, Wu X-X, Andree HA, Ross JB, Rusinova E, Gascon-Lema MG, Calandri C, Harpel PC. Antiphospholipid antibodies accelerate plasma coagulation by inhibiting annexin-V binding to phospholipids: a ‘lupus procoagulant’. *Blood*. 1998; 92:1652–1660. [PubMed: 9716593]
- Rand JH, Wu X-X, Guller S, Gil J, Guha A, Scher J, Lockwood CJ. Reduction of annexin-V (placental anticoagulant protein-I) on placental villi of women with antiphospholipid antibodies and recurrent spontaneous abortion. *Am. J. Obstet. Gynecol.* 1994; 171:1566–1572. [PubMed: 7802069]
- Rand JH, Wu XX, Lapinski R, van Heerde WL, Reutelingsperger CP, Chen PP, Ortel TL. Detection of antibody-mediated reduction of annexin A5 anticoagulant activity in plasmas of patients with the antiphospholipid syndrome. *Blood*. 2004; 104:2783–2790. [PubMed: 15242878]
- Rand JH, Wu X-X, Quinn AS, Ashton AW, Chen PP, Hathcock JJ, Andree HAM, Taatjes DJ. Hydroxychloroquine protects the annexin A5 anticoagulant shield from disruption by antiphospholipid antibodies: evidence for a novel effect for an old antimalarial drug. *Blood*. 2010a; 115:2292–2299. [PubMed: 19965621]
- Rand JH, Wu X-X, Quinn AS, Chen PP, Hathcock JJ, Taatjes DJ. Hydroxychloroquine directly reduces the binding of antiphospholipid antibody – β_2 -glycoprotein I complexes to phospholipid bilayers. *Blood*. 2008a; 112:1687–1695. [PubMed: 18577708]
- Rand JH, Wu X-X, Quinn AS, Chen PP, McCrae KR, Bovill EG, Taatjes DJ. Human monoclonal antiphospholipid antibodies disrupt the annexin A5 anticoagulant shield on phospholipid bilayers. Evidence from atomic force microscopy and functional assay. *Am. J. Pathol.* 2003; 163:1193–1200. [PubMed: 12937161]
- Rand JH, Wu X-X, Quinn AS, Taatjes DJ. Resistance to annexin A5 anticoagulant activity: a thrombogenic mechanism for the antiphospholipid syndrome. *Lupus*. 2008b; 17:922–930. [PubMed: 18827057]

- Rand JH, Wu X-X, Quinn AS, Taatjes DJ. The annexin A5-mediated pathogenic mechanism in the antiphospholipid syndrome: role in pregnancy losses and thrombosis. *Lupus*. 2010b; 19:460–469. [PubMed: 20353989]
- Reviakine I, Brisson A. Formation of supported phospholipid bilayers from unilamellar vesicles investigated by atomic force microscopy. *Langmuir*. 2000; 16:1806–1815.
- Reviakine I, Bergsma-Schutter W, Brisson A. Growth of protein 2-D crystals on supported planar lipid bilayers imaged *in situ* by AFM. *J. Struct. Biol.* 1998; 121:356–361. [PubMed: 9705879]
- Reviakine I, Bergsma-Schutter W, Mazeres-Dubut C, Govorukhina N, Brisson A. Surface topography of the p3 and p6 annexin V crystal forms determined by atomic force microscopy. *J. Struct. Biol.* 2000; 131:234–239. [PubMed: 11052896]
- Richter RP, Him JLK, Tessier B, Tessier C, Brisson AR. On the kinetics of adsorption and two-dimensional self-assembly of annexin A5 on supported lipid bilayers. *Biophys. J.* 2005; 89:3372–3385. [PubMed: 16085777]
- Rothfield N. Efficacy of antimalarials in systemic lupus erythematosus. *Am. J. Med.* 1988; 85(Suppl 4A):53–56. [PubMed: 3177431]
- Roubey AS, Eisenberg RA, Harper MF, Winfield JB. “Anticardiolipin” autoantibodies recognize β 2-glycoprotein I in the absence of phospholipid: importance of Ag density and bivalent binding. *J. Immunol.* 1995; 154:954–960. [PubMed: 7814895]
- Subang R, Levine JS, Janoff AS, Davidson SMK, Taraschi TF, Koike T, Minchey SR, Whiteside M, Tannenbaum M, Rauch J. Phospholipid-bound β 2-glycoprotein I induces the production of antiphospholipid antibodies. *J. Autoimmunity*. 2000; 15:21–32. [PubMed: 10936025]
- Taatjes DJ, Quinn AS, Lewis MR, Bovill EG. Quality assessment of atomic force microscopy probes by scanning electron microscopy: Correlation of tip structure with rendered images. *Microsc. Res. Tech.* 1999; 44:312–326. [PubMed: 10090206]
- Taatjes DJ, Sobel BE, Budd RC. Morphological and cytochemical determination of cell death by apoptosis. *Histochem. Cell Biol.* 2008a; 129:33–43. [PubMed: 18000678]
- Taatjes DJ, Wadsworth MP, Quinn AS, Rand JH, Bovill EG, Sobel BE. Imaging aspects of cardiovascular disease at the cell and molecular level. *Histochem. Cell Biol.* 2008b; 130:235–245. [PubMed: 18506469]
- Tait JF, Gibson D. Phospholipid binding of annexin V: effects of calcium and membrane phosphatidylserine content. *Arch. Biochem. Biophys.* 1992; 298:187–191. [PubMed: 1388011]
- Tait JF, Sakata M, McMullen BA, Miao CH, Funakoshi T, Hendrickson LE, Fujikawa K. Placental anticoagulant proteins: isolation and comparative characterization four members of the lipocortin family. *Biochemistry*. 1988; 27:6268–6276. [PubMed: 2975506]
- Tsakonas E, Joseph L, Esdaile JM, Choquette D, Senecal J-L, Cividino A, Danoff D, Osterland CK, Yeadon C, Smith CD. A long-term study of hydroxychloroquine withdrawal on exacerbations in systemic lupus erythematosus. *Lupus*. 1998; 7:80–85. [PubMed: 9541091]
- Van Genderen HO, Kenis H, Hofstra L, Narula J, Reutelingsperger CPM. Extracellular annexin A5: Functions of phosphatidylserine-binding and two-dimensional crystallization. *Biochimica. et Biophysica. Acta*. 2008; 1783:953–963. [PubMed: 18334229]
- Voges D, Berendes R, Burger A, Demange P, Baumeister W, Huber R. Three-dimensional structure of membrane-bound annexin V - a correlative electron microscopy-x-ray crystallography study. *J. Mol. Biol.* 1994; 238:199–213. [PubMed: 8158649]
- Wang SX, Cai GP, Sui SF. Intrinsic fluorescence study of the interaction of human apolipoprotein H with phospholipid vesicles. *Biochemistry*. 1999; 38:9477–9484. [PubMed: 10413525]
- Willems GM, Janssen MP, Comfurius P, Galli M, Zwaal RF, Bevers EM. Competition of annexin V and anticardiolipin antibodies for binding to phosphatidylserine containing membranes. *Biochemistry*. 2000; 39:1982–1989. [PubMed: 10684648]
- Wu XX, Guller S, Rand JH. Hydroxychloroquine reduces binding of antiphospholipid antibodies to syncytiotrophoblasts and restores annexin A5 expression. *Am. J. Obstet. Gynecol.* 2011; 205:576.e7–576.e14. [PubMed: 21871597]
- Zhu M, Olee T, Le DT, Roubey RAS, Hahn BH, Woods VL Jr, Chen PP. Characterization of IgG monoclonal anti-cardiolipin/anti- β 2-GPI antibodies from two patients with antiphospholipid

syndrome reveals three species of antibodies. Br. J. Haematol. 1999; 105:102–109. [PubMed: 10233371]

Figure 1.

Temporal formation of 2-D crystalline lattice of AnxA5 on planar lipid model membrane (PSPC) in the presence of BSA. (A) In the presence of BSA, the artificial planar lipid surface presents many defects (asterisks) with incorporated aggregates of BSA macromolecules. (B–F) AnxA5 coalescing into a 2-D crystal lattice over the planar lipid membrane from different nucleation sites as indicated by the opposing alignments of the molecular structure of AnxA5; the differently angled, corrugated appearance of the surface is indicative of the transitional phases AnxA5 demonstrates while it conformationally forms the 2-D crystal. In addition, the AnxA5 growth displays junctures termed furrows (white arrows); also referred to as grain boundaries the furrows are suspected to represent entry

locations for competing plasma proteins bound for lipid surface interactions. Note that the AnxA5 crystal forms around the defects. (G and H) illustrate completed 2-D crystal lattice formation of AnxA5 on the planar lipid layer and around the surface defects (BSA sites). The images in A-H are $5.0\ \mu\text{m} \times 5.0\ \mu\text{m}$ amplitude images electronically zoomed from $10.0\ \mu\text{m} \times 10.0\ \mu\text{m}$ original scans, with no off-line processing. Scan rate was 3.05 Hz. This figure is an electronically zoomed modification of Figure 15.2 previously published in Montigny et al. 2006.

Force Plots

Tip
Amplitude
2.60 nm/div
Setpoint—

Z position - 11.60 nm/div

Tip
Amplitude
2.60 nm/div
Setpoint—

Z position - 11.60 nm/div

Figure 2.

Typical force plots showing the interaction of a probe on the surface of mica and an artificial lipid bilayer (PSPC) in a physiological buffer containing 1.25 mmol/L CaCl_2 . The graphs present coinciding extension and retraction plots that display the hardness or softness of a surface. Force plot (A) insures a clean buffer and a compatible probe with no prominent adhesions resulting at the beginning of the imaging sessions and is indicative of the hard surface mica, and plot (B) displays a more rounded smooth data curve indicating a soft surface – i.e., a planar lipid surface.

Figure 3.

Sequential effects of plasma protein β_2 GPI and aPL mAb IS3 on AnxA5 crystal structure. (A–F) Temporal formation of an AnxA5 2-D crystal lattice on a planar phospholipid model membrane on muscovite mica. (A) “Pebbled” surface indicates primary conformational surface reaction and forming AnxA5 crystal (arrow) lattice. (B) Appearance of large and small irregular furrows (arrows) between coalescing AnxA5 lattices. (C) Higher magnification showing vacancies (arrow; small holes) potentially indicating incomplete crystal lattice formation. (D) Small furrows still exist after 35 minutes of continuous imaging. After 1 hour (E) and 6 hours (F) of continuous imaging, very few vacancies (arrow) remain. Note the crystal structure now readily apparent in (F). (G) Elevated nodules (arrows) demonstrate addition of β_2 GPI. The addition of β_2 GPI alone has no discernible effect on the integrity of the preformed crystalline lattice as observed at higher magnifications using height (E) and amplitude (F, G) images. (H, I) On the subsequent addition of mAb IS3, elevated globular structures, presumably antibody-antigen complexes (arrowheads) and circular deformities appeared, with disruption of the crystal lattice structure. In image (I), an x-y offset shows further extent of early stage reaction including furrow (elongated arrow) reappearance. Height images (A–E, H, I) were processed with third order flatten and erase scan line functions. Amplitude images (F, G) were processed with a 1X convolution. All images are 3-D views with 45° pitch and zero rotation (reproduced from Taatjes et al, 2008b).

Figure 4.

Control experiment demonstrating the lack of effect of β 2GPI followed by a control IgG1 antibody on the AnxA5 2-D crystalline lattice formed on a planar lipid layer. (A) Planar lipid layer with a few voids (arrows). (B) Initial steps in the formation of AnxA5 2-D crystal lattice represented by a “pebbled” surface appearance. (C, D) AnxA5 2-D crystal formation as the protein coalesces into a homogenous layer covering the lipid surface. Note that the protein does not bind to void areas in the planar lipid layer (arrows). (E) After sequential addition of β 2GPI and non-specific monoclonal antibody IgG1, structures (arrowheads) interact with surface of 2D crystal lattice. (F) Following overnight incubation, crystal lattice appears intact without disruption. β 2GPI clusters with some antibody attachments are

apparent. All panels depict zoomed original amplitude images. Scan line removal was performed on image (D) Scan rate was 3.05 Hz.

Figure 5. aPL mAb IS4 targets plasma protein β 2GPI after AnxA5 crystal structure formation. (A–F) AFM images from a dynamic fluid tapping mode imaging experiment showing mAb IS4 (indicated in yellow) targeting β 2GPI and illustrating disruption of AnxA5 2-D crystal structure 24 hours later. (A) β 2GPI monomers and oligomers on planar lipid model membrane. (B) Further oligomerization into an agglomeration of patch-like clusters (individual patches numbered 1–5). (C) Addition of AnxA5 results in 2-D crystal lattice formation over lipid membrane (large arrow indicates typical AnxA5 furrow). (D) Addition of aPL mAb IS4 (pseudocolored yellow for clarity) results in binding to clusters numbered 1–3. Raised ridge-lines (arrowheads) are at furrow locations and are likely to be residual

β 2GPI monomers and/or multimers that localize to border areas where AnxA5 crystallization may be incomplete. (E) Demonstration of continued binding and expansion of aPL mAb IS4 at respective β 2GPI cluster sites. (F) Following an overnight incubation at 4°C, the AnxA5 surface is disrupted. Amplitude images (A-E) and height image (F) represent 10 $\mu\text{m} \times 10 \mu\text{m}$ images electronically zoomed from 20 $\mu\text{m} \times 20 \mu\text{m}$ original scans. Scan rate was 3.05 Hz. Yellow pseudo-coloring for image enhancement detailing antibody binding to β 2GPI cluster sites was performed in Adobe PhotoShop (D,E).

Figure 6.

Temporal imaging session showing that aPL mAb IS3 targets β_2 GPI clusters and ridge-line sites, and over time degrades AnxA5 2-D crystalline lattice. (A) β_2 GPI clusters (numbered 1–6) on planar lipid layer. (B) Formation of AnxA5 2-D crystal lattice around clusters and ridge-lines of residually, weakly bound β_2 GPI. (C) Addition of aPL mAb IS3 results in binding to β_2 GPI at ridge line. Arrow indicates time at which antibody was added during an upward scan (line disturbance), and arrowheads indicate some antibody attachment sites. (D) Subsequent scan shows antibody-mediated surface degradation of AnxA5 2-D crystal lattice. Amplitude images (A–D) were electronically zoomed from original $30\ \mu\text{m} \times 30\ \mu\text{m}$ scans. Scan rate was 1.0 Hz.

Figure 7.

AnxA5 2-D crystal lattice formation on planar lipid layer is not prevented in the presence of β_2 GPI/IgG₁ and HCQ. (A) Planar lipid layer on cleaved muscovite mica surface. (B–F) Temporal demonstration of β_2 GPI binding and clustering on the planar lipid layer. (G) 18–24 hours following the addition of control IgG₁ and HCQ, large β_2 GPI clusters, possibly including some bound IgG₁ and HCQ are present. (H, I) Subsequent addition of AnxA5 results in 2-D crystal lattice formation and pushing non-tightly bound β_2 GPI to furrows as indicated by the ridge lines evident in (I). Amplitude images (A–I) are $30\ \mu\text{m} \times 30\ \mu\text{m}$ scans captured at 3.05 Hz scan rate and 90° scan angle.

Figure 8.

Atomic force microscopy (AFM) height images (3-dimensional view with 50° pitch) showing the effect of HCQ on aPL mAb and β_2 GPI complexes on 3 separate complexes. (A,E,I) The binding of β_2 GPI to phospholipid bilayers. Addition of β_2 GPI (10 μ g/mL) to buffer covering the phospholipid bilayers resulted in the formation of distinct structures raised above the bilayers. (B,F,J) The binding of aPL IgG mAb, IS4, to β_2 GPI prebound to phospholipids. Addition of aPL mAb (20 μ g/mL) to the phospholipid-bound β_2 GPI shown in panels A, E, and I resulted in large aggregates of increased height (indicated by white color) composed of aPL mAb- β_2 GPI complexes over the bilayers. (C,G,K) The effect of HCQ at 1 mg/mL on the phospholipid-bound aPL mAb and β_2 GPI complexes. At 12

minutes after the addition of HCQ to the supernatant fluid covering the bilayer, the immune complexes, shown in panels B, F and J, were significantly eroded after addition of the drug. (D,H,L) The immune complexes, shown in panels B, F, and J, were further disintegrated at thirty minutes after the addition of HCQ (1 mg/mL). All images show complexes electronically zoomed from an original $30\text{ }\mu\text{m} \times 30\text{ }\mu\text{m}$ scan. Images were minimally processed to remove scan lines. The color shades represent height variations in the images, with the darker colors (rust) indicating lower heights and the lighter colors (white), the higher profiles. (M) Quantitative analysis of the effects of HCQ on 40 randomly encountered aPL mAb- β_2 GPI complexes. The height of the immune complexes at 12 minutes after the addition of HCQ (1 mg/mL) was significantly decreased ($17.7 \pm 5.8\text{ nm}$; vs $9.3 \pm 2.4\text{ nm}$ for the height before addition of HCQ) (reproduced from Rand et al 2008a).

Figure 9.

Dynamic AFM imaging of HCQ-induced formation of AnxA5 layer over aPL IgG- β_2 GPI complexes. Each original AFM amplitude image (top panel) is paired with a colorized version (bottom panel) for explanation. In a sequence performed without HCQ, (A) the addition of β_2 GPI to phospholipid bilayers resulted in the formation of clusters (marked 1–4, orange circles in colorized version). (B) Addition of AnxA5 resulted in the initiation of 2-dimensional crystallization (flat round structures, green in colorized version) arising from different nucleation points, which (C) enlarge, merge, and grow toward each other until they covered all of the bilayer not occupied by the β_2 GPI cluster. (D) Subsequently added aPL mAb bound to the surfaces of β_2 GPI clusters. Note that in this image insufficient time had

elapsed for antibody-induced degradation of the AnxA5 crystal structure. In a sequence that included HCQ, (E) addition of β_2 GPI to phospholipid bilayers and formation of β_2 GPI clusters (marked 1–5) were followed by (F) addition of HCQ, followed by (G) addition of AnxA5, which at first crystallized from multiple different nucleation sites on the phospholipid bilayer, (H) and then formed a secondary layer of AnxA5 over the β_2 GPI, which progressively grew around and over the adjacent primary layer of AnxA5 crystal, leaving none of the surface uncovered by AnxA5 (reproduced from Rand et al 2010a).