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Review

Insights into the pathophysiology of the antiphospholipid syndrome provided by atomic force microscopy

Anthony S. Quinn[a], Xiao-Xuan Wu[b], Jacob H. Rand[b], Douglas J. Taatjes[a,*]

[a] Microscopy Imaging Center and Department of Pathology, College of Medicine, University of Vermont, Burlington, VT 05405, USA
[b] Department of Pathology, Montefiore Medical Center, Albert Einstein College of Medicine, Bronx, NY 10467, USA

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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_2GPI). Earlier, we hypothesized that the clinical manifestations arise from antibody-induced disruption of a two-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.

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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_2GPI) (Bevers et al., 1991; Oosting et al., 1993; de Laat et al., 2004, 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 laboratory modalities employed to diagnose and assess the molecular phenomenon (Galli et al., 2003; de Laat et al., 2004, 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 et al. (2004) have called for a universal standardization of the ELISA assay with respect to β_2GPI. Likewise, ellipsometry and coagulation tests (i.e., lupus anticoagulant (LA) tests) have provided much needed clinical

* Corresponding author.
E-mail address: douglas.taatjes@uvm.edu (D.J. Taatjes).

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information for APS, albeit accompanied by a lack of universal acceptance (Bevers et al., 2000; Willems et al., 2000). Investigating the antigenic determinants of β_2GPI 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_2GPI must interact with lipid before an aPL mAb will bind to the complex. Mechanistically, they proposed that a conformational change to β_2GPI 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_2GPI 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_2GPI 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_2GPI 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 aminoterminus. 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., 1996). 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_2GPI (Rand et al., 2003, 2008a). Our AFM studies have included observing the temporal and spatial interactions of AnxA5, β_2GPI, 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 immunomodulary drug hydroxycholoroquine (HCQ) on formed aPL mAb-β_2GPI 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 (1.25 mmol/l) of calcium chloride ($CaCl_2$); for example, as reported in earlier mechanistic studies of AnxA5-membrane interactions with other modalities (Tait and 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, 2006), biochemistry (Rand et al., 2003, 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 (Taatjes 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 min, 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 et al. (2002, 2004) validated