Micron 100 (2017) 23–29

Contents lists available at ScienceDirect

Micron

journal homepage: www.elsevier.com/locate/micron

Tutorial

Visualization of macro-immune complexes in the antiphospholipid syndrome by multi-modal microscopy imaging

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ARTICLE INFO

Keywords:
Antiphospholipid syndrome
Atomic force microscopy
Scanning electron microscopy
Macro-immune complexes

ABSTRACT

The antiphospholipid syndrome (APS) is an autoimmune thrombotic condition that is marked by autoantibodies against phospholipid-binding proteins. The mechanism(s) of thrombogenesis has (have) resisted elucidation since its description over thirty years ago. Nevertheless, a defining aspect of the disorder is positivity for clinical laboratory tests that confirm antibody binding to anionic phospholipids. It is remarkable that, to our knowledge, the binding of proteins from plasmas of APS patients to phospholipid has not been previously imaged. We therefore investigated this with high resolution microscopy-based imaging techniques that have not been previously used to address this question, namely atomic force microscopy and scanning electron microscopy. Atomic force microscopy imaging of APS plasmas incubated on an anionic planar phospholipid layer revealed the formation of distinct complex three-dimensional structures, which were morphologically dissimilar to structures formed from control plasmas from healthy patients. Likewise, scanning electron microscopy analysis of phospholipid vesicles incubated with APS plasmas in suspension showed formation of layered macro-immune complexes demonstrated by the significant agglomeration of a complex proteinaceous matrix from soluble plasma and aggregation of particles. In contrast, plasmas from healthy control samples bound to phospholipid vesicles in suspension generally displayed a more flattened, mat-like appearance by scanning electron microscopy. Scanning electron microscopy of plasma samples incubated on planar phospholipid layers and previously imaged by atomic force microscopy, corroborated the results obtained by mixing the plasmas with phospholipids in solution. Analysis of the incorporated proteins by silver stained SDS-polyacrylamide gel electrophoresis indicated considerable heterogeneity in the composition of the phospholipid vesicle-adsorbed proteins among APS patients. To our knowledge, these results provide the first images of plasma-derived APS immune complexes at high resolution, and show their consistent presence and heterogeneous compositions in APS patients. These findings demonstrate how high resolution microscopic techniques can contribute to advancing the understanding of an enigmatic disorder and may lay additional groundwork for furthering mechanistic understanding of APS.

1. Introduction

The antiphospholipid syndrome (APS) is a major thrombotic disorder that was described about 30 years ago (Hughes et al., 1986). The pathogenic mechanism(s) has (have) not been established. APS came to be recognized as a distinct clinical entity through astute clinical observations of laboratory anomalies. The discovery path began with the development of immunoassays for antibodies against cardiolipin, the key component of the classical serologic test for syphilis (Harris et al., 1983). Elevated levels of anticardiolipin antibodies were then found not to correlate well with syphilis, but rather with thrombosis and spontaneous pregnancy losses. Shortly thereafter, it was discovered that the major target of the autoantibodies in this assay was the phospholipid (PL)-binding proteins, β_2-glycoprotein I (β_2-GPI) and not cardiolipin itself (Galli et al., 1990; McNeil et al., 1990). APS is currently defined clinically by positive laboratory tests for anticardiolipin antibodies, anti-β_2-GPI antibodies, and the lupus anticoagulant (LA) phenomenon (Miyakis et al., 2006).

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http://dx.doi.org/10.1016/j.micron.2017.04.005
Received 11 April 2017; Received in revised form 19 April 2017; Accepted 21 April 2017
Available online 24 April 2017
0968-4328/

The pathogenic mechanism(s) of this disorder has (have) not yet been established (Rand et al., 2008b, 2010b). To approach the molecular mechanisms underpinning APS from a fresh perspective, we had previously imaged, by atomic force microscopy (AFM), the binding of monoclonal and purified immunoglobulins along with purified proteins to planar PL bilayers in an in vitro simulacrum (Rand et al., 2003, 2008a, 2010a; Montigny et al., 2006; Quinn et al., 2012a,b; Taatjes et al., 2013, 2014; Bezati et al., 2015); however, the interaction between components from APS plasmas and PL have not previously been reported. This is particularly significant since it has become clear that β_2-GPI is not the only target antigen in this disorder (Amiral et al., 1994). We therefore investigated the possibility that visible structures might be identified if the APS plasma-PL interaction were imaged with high resolution microscopy techniques – specifically AFM and scanning electron microscopy (SEM). These investigations led us to identify the presence of macro-immune complexes (macro-ICs) on (1) planar PL layers formed on mica discs, and (2) PL vesicles in suspension. Moreover, we sought to characterize their morphological appearance via two different processing protocols, and assess the homogeneity versus heterogeneity of cofactor proteins from individual patients.

2. Materials and methods

2.1. Plasmas and plasma-isolated antibodies

The research was approved by the institutional review board of Montefiore Medical Center, which granted permission for the use of anonymized excess plasmas from clinical assays or plasmapheresate discards (n = 6). De-identified normal healthy control plasmas (n = 5) were obtained from a commercial vendor that specializes in providing plasmas from carefully pre-screened normal healthy donors (George King Bio-Medical, Overland Park, KS).

2.2. Atomic force microscopy

The adsorption of proteins from APS plasmas to PL bilayers was observed by AFM in a similar manner to what had been previously described for the purified proteins (Rand et al., 2003; Quinn et al., 2012a,b). Briefly, PL vesicles (30% PS/70% PC) were sonicated twice on ice with an ultrasonic processor (Fisher Scientific) at 20% pulse for 20 s; the vesicles were then applied to freshly cleaved 1.2 cm mica discs (Ashville-Schoonmaker Mica Co., Newport News, VA), covered with HEPES-buffered saline, pH 7.4 (HBS), and planar PL bilayers were allowed to form on the disc surfaces. Plasma samples were then added to HBS covering the PL bilayer on the mica surface (final dilution 1:50) and stored overnight at room temperature to allow binding to occur. The following day, unbound plasma was rinsed away with buffer, and the sample was then covered with fresh buffer and observed using intermittent contact (AC) imaging with an Asylum Instruments MFP 3D-BIO AFM (Asylum Research, an Oxford Instruments company, Santa Barbara, CA) employing Pyrex Nitride Probes, silicon nitride SPM sensor (DNP-DB-20; Nanoworld, Neuchatel, Switzerland). Images in fluid were acquired at a scan rate between 0.3–1.0 Hz, at 512 × 512 pixel resolution. Five individual normal plasma samples were imaged between 1 and 7 times, while six individual APS plasma samples were imaged between 2 and 14 times.

2.3. Scanning electron microscopy

Plasma-treated PL vesicles were imaged with scanning electron microscopy (SEM) as follows. A mixture of 30% PS and 70% PC in chloroform was dried under a vacuum and re-suspended in HBS to a final concentration of 5 mM/mL. The vesicles formed were vortexed on a digital vortex mixer (Fisher Scientific) at 3000 rpm for 10 min; aliquots were stored at −20 °C and defrosted and vortexed for 5 min at 3000 rpm for SEM studies. PL vesicles (50 μL, 2 mM/mL), that were unsonicated to preserve vesicular structure, were incubated with patient-derived APS plasmas or normal healthy control plasmas (final 1:5 diluted in HBS) at room temperature for 2 h with constant mixing. For SEM preparation, the samples (N = 1 for each sample) (1) APS plasma + PL; (2) normal healthy control plasma + PL; and (3) PL alone with HBS were pelleted by centrifugation at 10,000 RPM for 2 min, followed by resuspension in fixative consisting of 2.5% glutaraldehyde/1.0% formaldehyde in 0.1 M cacodylate buffer for 1 h at 4 °C. Following 3 rinses (5 min each) with cacodylate buffer, the samples were post-fixed with 1% OsO_4 in cacodylate buffer for 1 h at 4 °C. Next, the samples were rinsed 3 times (5 min each) in cacodylate buffer, re-suspended by gentle vortexing, and deposited onto a 25 mm diameter Nucleopore Track-Etched membrane filter with 0.2 μm pore size (Fisher Scientific) inside of a EMD Millipore Swinnex-25 reusable filter holder (Fisher Scientific). The filters containing the samples were next dehydrated through a graded series of ethanol solutions (35%, 50%, 70%, 85%, 95%, and 100%) for 5 min at each concentration via a syringe attached to the filter holder. Finally, the filters were removed from the holder, air-dried overnight on a filter paper in a covered plastic dish, mounted onto aluminum SEM stubs with colloidal silver paint and sputter coated with gold/palladium. The filters containing the samples were then imaged with a JEOL 6060 scanning electron microscope (JEOL USA, Peabody, MA) operating at 20–25 kV.

2.4. Atomic force microscopy of samples followed by scanning electron microscopy

Some samples were imaged by AFM, followed by processing and viewing by SEM. Briefly, plasma samples from APS patients and normal healthy controls were deposited onto mica discs containing a planar PL layer and imaged by fluid-phase AFM as described above. After AFM imaging, the samples were fixed in 2.5% glutaraldehyde/1.0% formaldehyde in 0.1 M cacodylate buffer for 30 min at 4 °C, rinsed with cacodylate buffer, post-fixed in 1% OsO4 in cacodylate buffer for 30 min at 4 °C, rinsed in buffer, dehydrated in an ethanol series, mounted with colloidal graphite paint onto aluminum SEM stubs, sputter coated with gold/palladium, and viewed in a JEOL 6060 SEM, all as described above.

2.5. SDS-PAGE

PL vesicles consisting of 30% PS and 70% PC (50 μl, 2 mM) were incubated with 0.5% BSA for 30 min at room temperature, washed 3 times with HBS and re-suspended in HBS (50 μl). The vesicles were then incubated with APS and control plasmas (100 μl) in HBS (400 μl) at room temperature for 10 min, followed by 3 washes with HBS. The suspensions containing the vesicles were then incubated with Laemmli sample buffer (Bio-Rad Laboratories, Inc, USA) at a 1:1 ratio for 5 min at room temperature and loaded, at 20 μl/well each, on mini-protean TGX precast gels (Bio-Rad Laboratories). SDS-PAGE was then performed under non-reducing conditions and the proteins were detected with a Pierce Silver Stain kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The identities of three of the protein bands – IgG, β_2-GPI, and Apolipoprotein A1 (ApoA1) were confirmed by standard western blot procedures with specific antibodies for each of those proteins. Briefly, equivalent gels that had been electrophoresed as described above were washed in running buffer, transferred to a PVDF membrane according to manufacturer's instructions (Mini *trans*-Blot, Bio-Rad) and incubated with the primary and secondary antibodies, described below, followed by staining with tetramethylbenzidine (TMB) staining (1-Step Ultra TMB-Blotting solution; Pierce Biotechnology) according to the manufacturer's instructions. The primary antibodies were (1) rabbit polyclonal antibody against human IgG (H + L) (Thermo Scientific Pierce), (2) rabbit polyclonal antibody against full length native ApoA1 (Abnova), (3) polyclonal goat antihu-