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Full Length Article

Inverted erythrocyte membranes demonstrate β2GPI-antiphospholipid antibody interactions and membrane crosslinking

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ABSTRACT

Introduction: The antiphospholipid syndrome (APS) is an acquired autoimmune disorder predisposing patients to thrombosis or pregnancy complications. Since inverted erythrocyte membranes (iEMs) might provide a physiologically relevant source of anionic phospholipids, we studied the interactions of phospholipid-binding proteins and APS antibodies using iEMs.

Materials & methods: iEMs were prepared from packed erythrocytes by hypotonic lysis. Phosphatidylserine (PS) exposure was confirmed by annexin A5 (A5) binding using fluorescence microscopy and flow cytometry. Binding of β_2-glycoprotein I (β_2GPI)–IgG immune complexes to iEMs was investigated with gel electrophoresis, western blot and flow cytometry. Functional involvement in coagulation was documented in the thrombin generation assay.

Results: iEMs readily precipitated purified β_2GPI as well as β_2GPI from normal plasma and APS plasma. The plasma of APS patients provided higher levels of IgG binding to iEMs relative to healthy controls. Thrombin generation increased with increasing concentrations of iEMs, documenting that coagulation proteins bound to the exposed phospholipids. The LA effect was also distinguished in thrombin generation when comparing APS patients, as indicated by an increased lag time. Agglutination was observed after incubation with APS patient plasma and this was augmented by anti-human globulin.

Conclusions: In conclusion, iEMs can provide a more physiological approach than phospholipid vesicle-based tests for investigating APS and are more amenable to standardization than platelet membranes.

1. Introduction

The antiphospholipid syndrome (APS) is an acquired autoimmune disorder predisposing patients to venous and arterial thrombosis as well as pregnancy complications. Patients have circulating antibodies against proteins that bind to anionic phospholipid surfaces such as β_2-glycoprotein I (β_2GPI) and prothrombin [1–3]. Serological confirmation of the disorder is currently based on the detection of antibodies (IgG or IgM) against β_2GPI or against cardiolipin or the presence of lupus anticoagulant (LA). Clinical confirmation of APS is based on the presence of either vascular thrombosis (one or more episodes of venous, arterial or small vessel thrombosis) or pregnancy morbidity (miscarriages, premature births or unexplained fetal deaths). A diagnosis is only confirmed when patients are positive for one of the laboratory tests on at least two occasions over a period of 12 or more weeks and when they meet one of the clinical criteria [4].

β_2GPI is a phospholipid binding protein of about 50 kDa with a high plasma concentration of 50–400 μg/ml [5] and has been identified as the major target for APS antibodies. β_2GPI changes conformation after binding anionic phospholipids, exposing a neo-epitope domain I. Antibodies against this domain appear to be more commonly associated with thrombosis [6].

Typically, synthetic or isolated phospholipids are used to test the interaction of APS antibodies with β_2GPI and phospholipid surfaces. APS antibodies readily bind platelets [7,8], however the standardization of platelets as a phospholipid surface in APS research has been challenging. In this study we investigated whether a more physiological system using cell membranes as a source of anionic phospholipids could be suitable to study the interaction of β_2GPI and APS antibodies. In normal conditions, red blood cells (RBCs) present a limited amount of anionic phospholipids on their surface. The outer leaflet is mainly composed of sphingomyelin and phosphatidylcholine, while the inner leaflet consists largely of phosphatidylserine (PS) and phosphatidylethanolamine [9]. The exposure of negatively charged phospholipids might increase with aging. It was shown previously, that RBCs treated with and maintained in hypotonic media will become devoid of intracellular content.

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The inner membrane leaflet becomes exposed and a free membrane edge develops around the openings caused by the hypotonic lysis and some RBC ghosts will evert completely [10]. Additionally, it has been demonstrated that phospholipids are scrambled during hypotonic lysis due to direct connections between the inner and outer leaflets around the ruptured membrane edges. The absence of aminophospholipid translocase activity in RBCs prevents a rapid redistribution of aminophospholipids back to the inner monolayer [11,12]. These altered membrane shapes can persist for hours at low temperatures and are termed inverted erythrocyte membranes (iEMs).

We investigated the utility of iEMs as a phospholipid surface for APS by detecting exposure of anionic phospholipids, recognition by APS IgG, utility for thrombin generation, recognition of LA effect and binding of coagulation factors by comparing plasma samples of APS patients and healthy donors, thus developing a new model to study and further characterize APS.

2. Materials & methods

2.1. Reagents

Oregon green-conjugated annexin A5 (A5-OG) was from Molecular Probes (Eugene, OR, USA). Annexin A5 labelled with Alexa Fluor 647 (A5-Alexa 647) and polyclonal goat anti-human IgG-Alexa Fluor 633 (anti-hIgG Alexa 633) were from Life Technologies (Carlsbad, CA, USA). Anti-glycophorin A (anti-GYPA) monoclonal antibodies, clone HIR2, labelled with fluorescein isothiocyanate (FITC) were acquired from Abnova (Taipei, Taiwan). Polyclonal anti-β_2GPI was from R&D systems (Minneapolis, MN, USA). Rabbit anti-human IgG (Heavy + Light chain (H + L)) and goat anti-rabbit IgG (H + L) labelled with horseradish peroxidase (HRP) were from Thermo Fisher (Rockford, IL, USA). Rabbit anti-goat IgG (H + L)-HRP was from Calbiochem (EMD Millipore, San Diego, CA, USA). β_2GPI was from Genway Biotech Inc. (San Diego, CA, USA). Albumin was from Grifols Biologicals Inc. (Los Angeles, CA, USA). Anti-D series 4 (mouse monoclonal blend) was from Immucor (Norcross, GA, USA). Anti-human globulin (anti-human IgG/-C3d) and RhD positive RBC reagent cells were from Ortho Clinical Diagnostics (Raritan, NJ, USA). Expired RBC units, were obtained from the blood bank. The tissue factor (TF) used was Innovin (Dade Behring, Marburg, Germany).

2.2. Patient plasma samples

Plasma samples were collected in citrate tubes (3.2%) from confirmed APS patients and healthy donors in accordance with the Helsinki declaration and institutional IRB approval. All 15 patients from which samples were used during this study were LA positive. Five of these patients were positive for anti-cardiolipin and anti-β_2GPI antibodies, one patient was anti-cardiolipin IgG and IgM positive as well as anti-β_2GPI IgG positive, one patient was anti-cardiolipin IgM positive and one patient was anti-β_2GPI IgM positive. Plasmas were filtered with 0.2 μm syringe filters.

2.3. iEM and sample preparation

Erythrocyte ghosts were prepared from RBCs by hypotonic lysis in 20 mOsm (11.1 mM) Tris buffer using a modified version of earlier published protocols [10,13,14]. RBCs for iEMs were prepared from expired RBC units (over 28 days old) from the blood bank that were collected in ADSOL, irradiated and leukocyte reduced. The latter steps were included in order to minimize the possibility of iEM aggregation during lysis and high-speed centrifugation. Prior to lysis RBCs were washed twice in 0.9% NaCl followed by a third washing step in 172 mM Tris. RBCs were lysed in hypotonic Tris buffer and subsequently washed and maintained in 11.1 mM Tris buffer, pH 8.0 at 4 °C. iEMs were used fresh, stored at 4 °C for <7 days, or frozen-thawed after aliquoting and storage at −80 °C (iEMs were only frozen–thawed once). All experiments were performed with O RhD negative or RhD positive RBCs. All plasmas were confirmed not to contain anti-RhD.

2.4. Fluorescent microscopy

PS exposure on iEMs was documented using a fluorescence microscope equipped with a camera and Cytovision software (Leica Biosystems Inc., Buffalo Grove, IL, USA). iEMs were stained with 5 μg/ml A5-OG in 0.2% NaCl containing 5 mM $CaCl_2$.

2.5. Flow cytometry

PS exposure was also documented using flow cytometry (Becton Dickinson LSRII device (Franklin Lakes, NJ, USA)). A5-Alexa 647 was used in a 1/400 dilution in the presence of 5 mM $CaCl_2$ (in 0.2% NaCl) staining of iEMs was detected after 10 min incubation. An anti-GYPA antibody labelled with FITC was used to detect iEMs. Flow cytometry analyses were performed using sequential gating and standard quadrant analysis. Gating for background was based on the sample in the presence of A5-Alexa 647 and 20 mM EDTA. Median fluorescence intensities (MFI) were documented.

In order to detect IgG binding to iEMs, they were incubated with healthy or APS patient plasma for a minimum of 10 min in 0.2% NaCl at 4 °C, followed by consecutive washing steps by centrifugation at 15,000*g*. As controls for the plasma samples iEMs in 0.2% NaCl or incubated with 2.5% albumin were included. Binding of IgG to iEMs was investigated by flow cytometry using goat anti-H IgG-Alexa 633 in a 1/800 dilution incubated for 30 min. IgGs of patients and healthy donors were purified as described earlier [15].

2.6. Agglutination

Agglutination experiments were performed by the standard tube method using either iEMs or RBC reagent cells. Bright field and phase contrast images (400× magnification) of agglutination were obtained with an Olympus CKX41 microscope equipped with a camera (Olympus DP72) and cell Sens Entry software (Olympus, Center Valley, PA, USA). Additionally, indirect anti-human globulin testing was performed (with anti-human IgG/-C3d) as previously described [16].

2.7. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

For gel electrophoresis, SDS-PAGE, 4–15% TGX gels (Mini-Protean) from Bio-Rad were used with Tris/glycine/SDS buffer (Bio-Rad) as running buffer. iEMs were incubated with plasma samples and subsequently washed in 0.2% NaCl. These samples were diluted 2 times with Laemmli buffer (Bio-Rad) with or without 5% β-mercaptoethanol and heated to 95 °C for 5 min. A known, high molecular weight marker was included in each gel. Gels were stained with Coomassie Blue solution (1.25 g Coomassie Blue per liter solution containing 50% methanol, 7% glacial acetic acid) for 20 min. Gels were destained with a 10% acetic acid solution. Samples were initially evaluated under both reducing and non-reducing conditions. Analysis of protein bands density was performed with Gelanalyzer software version 2010a. α-Spectrin, present in the iEMs, was used to correct the density for loading variations.

2.8. Western blot

Gels for western blot were washed in running buffer and transferred to a PVDF membrane according to manufacturer's instructions (Mini Trans-Blot, Bio-Rad). The membranes were washed in washing buffer (0.1% Tween 20 in HBS) and incubated in blocking buffer (1% BSA in washing buffer) for 1 h. Following a washing step, the membranes were incubated with the primary antibody (R anti-H IgG or G anti-H