Sulfatides
Targets for Anti-Phospholipid Antibodies

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Background—Sulfatides are sulfated glycosphingolipids expressed on the surface of erythrocytes, leukocytes, and platelets. Sulfatides interact with several cell adhesion molecules involved in hemostasis. β2-Glycoprotein I is an anionic phospholipid-binding plasma protein, and the phospholipid-bound form is the target for most anti-phospholipid antibodies that are associated with recurrent thrombosis, miscarriages, and neurological symptoms. In this study, we examined whether β2-glycoprotein I forms a complex with sulfatides and thereby becomes a target for anti-phospholipid antibodies.

Methods and Results—β2-Glycoprotein I binds to surface-bound sulfatides but not to other glycolipids, such as ceramide, cerebrosides, sphingomyelin, or ganglioside. At a sulfatide coating density of 1 μg/well, β2-glycoprotein I reaches half-maximal binding at 2.5 μg/mL, and the binding is saturated at 10 μg/mL. The binding of β2-glycoprotein I also depends on the coating density of sulfatides in the well. At a constant β2-glycoprotein I concentration of 5 μg/mL, maximal binding of β2-glycoprotein I is observed at a coating density of 1 μg/well. The serum from 14 patients with anti-cardiolipin antibodies, a subset of anti-phospholipid antibodies, bound to sulfatide-bound β2-glycoprotein I and previous absorption on cardiolipin-coated surfaces decreased the immunoreactivity toward sulfatide-β2-glycoprotein I complex by >50% in 12 of 14 patients. Furthermore, immunoaffinity-purified anti-cardiolipin antibodies from 4 of 5 patients reacted with sulfatide-bound β2-glycoprotein I.

Conclusions—These results show that not only anionic phospholipids, as commonly known, but also sulfatides are targets for most anti-phospholipid antibodies. We therefore postulate that interactions of these antibodies with sulfatides may contribute to some of the clinical symptoms of the anti-phospholipid antibody syndrome. (Circulation. 2003;108:2082-2087.)

Key Words: glycoproteins ■ sulfatides ■ antibodies ■ coagulation

Sulfatides, galactosyl-3'-sulfate ceramides, are acidic glycosphingolipids containing sulfate esters on their oligosaccharide chains. In mammals, sulfatides are present primarily in nervous tissue, kidney, testis, erythrocytes, platelets, and granulocytes.1 In addition, it has been shown that sulfatides are one of the major lipids in serum.2 Sulfatides interact with a variety of cell adhesion molecules involved in hemostasis, such as von Willebrand factor,3 thrombospondin,4 selectins,5,6 and laminin.7 Furthermore, sulfatides activate plasma coagulation factor XII.8,9

β2-Glycoprotein I, also known as apolipoprotein H, is a 50-kDa plasma protein present at a concentration of ~200 μg/mL in human plasma.10 It belongs to the so-called “complement control protein (CCP)” or “sushi” domain superfamily, members of which are identified by the presence of one or more motifs containing a characteristic disulfide bond pattern.10,11 β2-Glycoprotein I is composed entirely of 5 CCP repeats. The fifth repeat, CCP5, diverges from the norm for CCPs in that it has a relatively unusual pattern of 3 disulfide bridges and contains a positively charged sequence, CKNKEKKC (residues 281 to 288), that mediates its binding to anionic phospholipids.11 By this interaction, β2-glycoprotein I is thought to play a role in the clearance of anionic phospholipid–containing vesicles and cell membranes from the circulation.12 When in complex with these phospholipids, β2-glycoprotein I is also a target for most anti-phospholipid antibodies associated with thrombosis.13,14

Here, we show that β2-glycoprotein I binds to sulfatides at physiological concentrations. Furthermore, β2-glycoprotein I–sulfatide complexes are recognized by most antibodies from patients with antiphospholipid antibody associated with thrombosis.

Methods

The reagents bovine brain sulfatides, phosphatidylserine, phosphatidylcholine, sphingomyelin (from brain), ceramide (brain), and cho-
lesterol were obtained from Avanti Polar Lipids. All other standard chemicals were obtained from Fisher chemical company.

Isolation of Human $\beta_2$-Glycoprotein I

$\beta_2$-Glycoprotein I was isolated from normal citrated plasma as described previously with some modifications. Plasma (100 mL) was mixed with 2.5 mL 70% (vol/vol) perchloric acid, stirred gently at 4°C for 15 minutes, and centrifuged at 13 000g for 15 minutes at 4°C. The supernatant was neutralized to pH 7.0 with 12.5 mol/L NaOH, and 43 g of ammonium sulfate powder was added slowly. The mixture was stirred at 4°C for 30 minutes and centrifuged at 13 000g for 15 minutes at 4°C. The precipitate was dissolved in 0.03 mol/L NaCl/20 mmol/L Tris-HCl, pH 8.0, and dialyzed against the same buffer. The sample was applied to a column of heparin-Sepharose (2×15 cm), which was washed sequentially with 400 mL 0.05 mol/L NaCl/20 mmol/L Tris, pH 8.0, and 400 mL 0.15 mol/L NaCl/20 mmol/L Tris, pH 8.0, and then eluted with 0.35 mol/L NaCl/20 mmol/L Tris, pH 8.0. The $\beta_2$-glycoprotein I–containing peak was dialyzed against HEPES-buffered saline (HBS) (0.05 mol/L HEPES, 0.05 mol/L NaCl, pH 7.4), applied to a Mono S column, and eluted with 0.05 to 0.5 mol/L salt gradient in 0.05 mol/L HEPES, pH 7.4. The $\beta_2$-glycoprotein I peak was collected and dialyzed against HBS (0.15 mol/L NaCl/0.02 mol/L HEPES, pH 7.4). A polyclonal antibody to $\beta_2$-glycoprotein I was prepared in rabbit by immunization with purified human $\beta_2$-glycoprotein I and was monospecific to $\beta_2$-glycoprotein I when tested against human plasma in Ouchterlony’s immunodiffusion and in immunoblots.

Sulfatide Binding Assays

The wells of a 96-well microtiter plate (Maxisorp P96, Nunc) were coated with sulfatides or other lipids (1 $\mu$g/well) by evaporating 100 $\mu$L of methanol solutions of lipids in the wells. The wells were blocked with 5% BSA in Tris-buffered saline (TBS: 10 mmol/L Tris, 150 mmol/L NaCl, pH 7.5). Various concentrations of $\beta_2$-glycoprotein I in TBS containing 1% BSA were added to the wells. After 2 hours, the wells were washed 3 times with TBS. Lipid-bound $\beta_2$-glycoprotein I was detected with a rabbit anti-human $\beta_2$-glycoprotein I antibody (1:1000), peroxidase-labeled protein A (1:1000), and O-phenylenediamine as a substrate. Optical density was measured in an ELISA plate reader at 450 nm. For dose-response isotherms of sulfatides, the wells were coated with various amounts of sulfatides (0.001 to 10 $\mu$g) onto the ELISA plates and the bound $\beta_2$-glycoprotein I protein was detected as above, after incubation with 100 $\mu$L of 5 $\mu$g/mL $\beta_2$-glycoprotein I.

Vesicle Aggregation Assay

Sulfatide vesicles (1 mmol/L) were prepared with a molar ratio of phospholipid to cholesterol of 1:0.75. The vesicles contained either 100% phosphatidylcholine (phosphatidylcholine vesicles) or a mixture of 50% phosphatidylcholine and 50% sulfatides (sulfatide vesicles). The lipid mixture was mixed in a glass ampule and dried to a thin film under a gentle stream of dry nitrogen. The dried lipid mixture was then mixed in a glass ampule and dried to a thin film under a gentle stream of dry nitrogen. The dried lipid mixture was then resuspended in HBS, and the ampoule was sealed and vesiculized in bath sonication (Bronson 3200, Laboratory Supplies Inc) for 45 minutes at 20°C. The vesicle suspension was cleared from large multilamellar liposomes by filtering in 0.2-$\mu$m filters. Changes in vesicle size were measured in samples containing 50 $\mu$L of phospholipids in a 2.5-mL volume by monitoring the absorbance at 320 nm as a function of time on a Beckman DU-8 spectrophotometer. In this assay, the turbidity of a suspension varies as a function of particle size, and no angular dependence is involved.

Patient Characteristics

We studied 14 patients with anti-cardiolipin antibodies and thromboembolic complications. Inclusion in this study was based on a high antibody titer against phospholipid antigens. Thromboembolic complications included deep venous thrombosis, arterial thrombosis, recurrent spontaneous abortion (defined as ≥2 fetal losses after 12 weeks of gestation), cerebrovascular thrombosis, and neurological symptoms (defined as partial complex seizures, multi-infarct dementia, chorea, or intractable migraine headaches) that could not be explained by any other cause. The clinical data are summarized in Table 1. Blood samples were collected in a 1/10 volume of trisodium citrate after informed consent approved by the institutional committee for the protection of human subjects.

ELISA for Anti-Phospholipid Antibodies

Flat-bottom non–tissue culture–treated polystyrene microtiter plates (Falcon 3912) were coated with 1 $\mu$L of cardiolipin or sulfatide as described previously. Plates were blocked with 10% FCS, which also provides $\beta_2$-glycoprotein I, for 1 hour and subsequently washed 4 times with TBS. Serum was diluted serially with TBS containing 1% BSA and allowed to incubate in the coated wells at room temperature for 1 hour. Plates were then washed 4 times with TBS, and the bound antibodies were detected with horseradish peroxidase–labeled rabbit anti-human immunoglobulins and the peroxidase substrate O-phenylenediamine (1 mmol/L) and H$_2$O$_2$.

Imunoaffinity Purification of Anti-Cardiolipin Antibodies

Anti-cardiolipin antibodies were purified as described by Pengo et al. Briefly, cardiolipin liposomes were prepared from a mixture of cardiolipin, cholesterol, and dicetyl phosphate (molar ratio, 10:15:1). Serum (5 $\mu$L) was incubated with liposomes (1 mg cardiolipin/mL serum) for 1 hour at room temperature, and the liposomes were washed with TBS. The liposome-bound antibodies were eluted by incubating the liposomes in 1 mol/L NaCl, dialyzed in TBS, and tested in ELISA for reactivity toward cardiolipin and sulfatide with 10% FCS as the blocking agent.

Absorption of Plasma on Cardiolipin Surface

Twenty-four–well polystyrene tissue culture plates (Greiner Bio-one) were coated with 5 $\mu$L of cardiolipin in 100 $\mu$L of ethanol and allowed to evaporate in a hood. The plates were blocked in 10% FCS, washed, and then incubated with 1:100 dilutions of plasma for 30 minutes at room temperature. The absorbed plasma samples were tested in ELISA for cardiolipin and sulfatide. The results were expressed as percentage of binding of the plasma before absorption with cardiolipin, which was considered to be 100%.
We examined the binding of β2-glycoprotein I to various lipids. As shown in Figure 1A, β2-glycoprotein I bound to surface-bound sulfatide in a saturable manner. At a sulfatide coating density of 1 μg/well, β2-glycoprotein I reached half-maximal binding at 2.5 μg/mL and saturated binding at 10 μg/mL. In contrast, under similar conditions, β2-glycoprotein I did not bind significantly to other glycolipids, such as ceramide, cerebrosides, sphingomyelin, or gangliosides, under similar conditions (Figure 1B). As shown previously, β2-glycoprotein I also bound to cardiolipin and other anionic phospholipids but not to the neutral or zwitterionic phospholipids (Figure 1B). The extent of β2-glycoprotein I binding also depended on the coating density of sulfatides (Figure 1C). With a β2-glycoprotein I concentration of 5 μg/mL, maximal binding of β2-glycoprotein I was observed at a sulfatide coating density of 1 μg/well. To assess the nature of β2-glycoprotein I binding to sulfatides, we examined the binding at different salt concentrations. The binding decreased with increasing salt concentrations, suggesting that a significant component of the binding is caused by electrostatic interactions (Figure 2).

**Results**

**β2-Glycoprotein I Binds to Sulfatide**

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**Plasma β2-Glycoprotein I Binds to Sulfatides**

Several sulfatide-binding proteins are present in plasma, such as von Willebrand factor, thrombospondin, and P-selectin.1–6 To determine whether β2-glycoprotein I will bind sulfatides in the plasma milieu despite the presence of other sulfatide I–binding proteins, we incubated plasma in different dilutions on sulfatide-coated surfaces and measured the binding of plasma β2-glycoprotein I to sulfatides. As shown in Figure 3, there was a concentration-dependent binding of β2-glycoprotein I to sulfatide, showing that this interaction occurs also in the plasma milieu.
Interaction of Cardiolipin-Dependent
\(\beta_2\)-Glycoprotein I Autoantibodies With
Sulfatide-Bound Cardiolipin

To determine whether the same antibodies reacting with cardiolipin-\(\beta_2\)-glycoprotein I complex also react with sulfatide-\(\beta_2\)-glycoprotein I complex, we absorbed the serum from the 14 patients on a cardiolipin-coated surface. Immunoreactivity toward sulfatide-\(\beta_2\)-glycoprotein I complex decreased by \(>50\%\) in 12 of 14 patients tested (Figure 6). In 1 patient (patient 5), there was no reduction in reactivity to sulfatide-\(\beta_2\)-glycoprotein I despite a 70% reduction in reactivity to cardiolipin-\(\beta_2\)-glycoprotein I complex (Figure 6). Furthermore, we affinity-purified the anti-cardiolipin antibodies on cardiolipin liposomes from 5 patients in whom we had sufficient plasma. We tested the immunoaffinity-purified antibodies in ELISA against cardiolipin- and sulfatide-\(\beta_2\)-glycoprotein I complexes. In 4 of 5 patients, the antibodies reacted with sulfatide-\(\beta_2\)-glycoprotein I complex. In 1 patient, despite significant reaction to cardiolipin, no reactivity to sulfatide was seen (Figure 7, patient 2). Interestingly, patient 2, in whom the immunoaffinity-purified anti-cardiolipin antibodies had no reactivity to sulfatide-\(\beta_2\)-glycoprotein I complex, still had a modest 30% reduction in reactivity in absorption experiments (Figure 6). This may be because of conformational differences in epitope(s) presented by liposome-bound versus immobilized surface-bound cardiolipin-\(\beta_2\)-glycoprotein I complexes. Taken together, these findings show that the majority of the anti-phospholipid antibodies reacting with cardiolipin-\(\beta_2\)-glycoprotein I complex also react with the sulfatide-\(\beta_2\)-glycoprotein I complex. However, in at least some patients, there are separate antibodies specific to each of these complexes.

Discussion

Sulfatides have been proposed as modulators of the hemostatic process. They not only activate the contact phase of the coagulation cascade but also provide substrates for several adhesion molecules of hemostasis. Recently, we have shown that sulfatides are also involved in platelet adhesion and aggregation as receptors for P-selectin.\(^{19}\)

\(\beta_2\)-Glycoprotein I is a plasma protein that binds negatively charged surfaces, including anionic phospholipid vesicles and platelets, and this interaction inhibits platelet- and phospholipid-dependent coagulation reactions.\(^{18,20}\) Furthermore, Chonn et al\(^{12}\) identified \(\beta_2\)-glycoprotein I as a major

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**Figure 4.** Effect of \(\beta_2\)-glycoprotein I on turbidity of sulfatide vesicles. Time course of increase in turbidity as a measure for aggregation of sulfatide vesicles was monitored at \(\lambda\) 320 nm. Aggregation of sulfatide vesicles was induced by various concentrations of \(\beta_2\)-glycoprotein I (●, 1 \(\mu\)g/mL; ■, 2 \(\mu\)g/mL; ●, 5 \(\mu\)g/mL; and ○, 10 \(\mu\)g/mL. Phosphatidylcholine vesicles were used as controls with 10 \(\mu\)g/mL of \(\beta_2\)-glycoprotein I (○).

**Figure 5.** Binding of anti-cardiolipin antibodies to sulfatides: ELISA plates were coated with 1 \(\mu\)g/well of cardiolipin (open bar) or sulfatides (closed bar) and blocked with 10% FCS, which also provides \(\beta_2\)-glycoprotein I. Serum (1:100 dilutions) from patients with anti-cardiolipin antibodies was added, and bound antibodies were measured with rabbit anti-human Ig. Binding of antibodies is expressed as ratio over normal pooled serum.
protein that binds to liposomes with a short half-life in vivo and noted that pretreating mice with anti-β2-glycoprotein I antibodies markedly increased the circulating half-life of the liposomes. In vitro, β2-glycoprotein I promotes the uptake of anionic phospholipid vesicles and platelet-derived microvesicles by macrophages.15,21 These studies suggest a role for β2-glycoprotein I in the clearance of anionic phospholipid-containing surfaces, such as activated platelets, microvesicles, and apoptotic cells with anionic phospholipid on their outer surface.

Our results show that β2-glycoprotein I binds to sulfatides as well as to anionic phospholipids, even in the presence of other sulfatide-binding proteins in plasma. Because the concentration of β2-glycoprotein I in plasma is much higher than the concentration of the other known sulfatide-binding protein, as the major sulfatide-binding protein, it may modulate sulfatide functions in hemostasis.

Lupus anticoagulants and anti-cardiolipin antibodies are immunoglobulins that interact with anionic phospholipids, namely phosphatidylserine, phosphatidic acid, phosphatidylinositol, and cardiolipin.22,23 Lupus anticoagulants are detected in coagulation assays, in which they prolong coagulation tests, whereas anti-cardiolipin antibodies are detected by immunologic assays. Interaction of these antibodies with anionic phospholipids in these assays depends on their binding to anionic phospholipid-binding proteins, of which β2-glycoprotein I is the major one. The clinical significance of the anti-phospholipid antibodies is related to their association with thromboembolic events, arterial, venous, embolic, and microvascular22,23 although no mechanism has been elucidated. Our results suggest that the target specificity of antibodies in the plasma of patients with anti-phospholipid syndromes is much broader than previously suspected, because the majority of the antibodies also react efficiently with sulfatide-bound β2-glycoprotein I. Furthermore, some of these patients also have an antibody specific for each of these complexes. These findings raise the possibility that the reaction of these antibodies with sulfatide may contribute not only to the hemostatic abnormalities but also other clinical features, such as the neurological symptoms and abortions, because sulfatides are present in nerve tissue1 and in the female genital tract.24

In summary, our results show that sulfatide–β2-glycoprotein I complexes are targets for anti-phospholipid antibodies, and these interactions may contribute to the clinical symptoms of the antiphospholipid syndrome.
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