Cholesterol Sulfate
A New Adhesive Molecule for Platelets

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Background—Cholesterol 3-sulfate is present on a variety of cells and in human LDL, and it has been found in atherosclerotic lesions of human aorta. Its precise biological role has not yet been described.

Methods and Results—In this study, we investigated the interaction of platelets with cholesterol sulfate. Platelets adhered in a concentration-dependent and saturable manner to cholesterol sulfate but did not adhere to cholesterol, cholesterol acetate, estrone sulfate, or dehydroepiandrosterone sulfate, suggesting that the specificity of this interaction is determined not only by the cholesterol moiety but also by the sulfate group. This adhesion did not increase after platelet activation, and it was not cation-dependent. Soluble cholesterol sulfate inhibited adhesion in a concentration-dependent manner. However, antibodies against glycoprotein Ib, glycoprotein IIb/IIIa, CD36, P-selectin, von Willebrand factor, or thrombospondin had no significant effect on platelet adhesion to cholesterol sulfate. Perfusion of whole blood in a parallel-plate flow chamber resulted in the rapid and progressive adhesion of platelets to cholesterol sulfate but not to cholesterol acetate or estrone sulfate.

Conclusions—Cholesterol sulfate supports platelet adhesion and may be one of the factors determining the prothrombotic potential of atherosclerotic lesions. (Circulation. 2001;103:2032-2034.)

Key Words: atherosclerosis ■ thrombosis ■ cell adhesion molecules

Cholesterol sulfate is widely distributed in various body fluids and in tissues and cells, including erythrocytes, platelets, skin, hair, adrenals, lung, and brain. Furthermore, it is present in plasma, LDL, and the atherosclerotic lesions of human aorta. The biological role of cholesterol sulfate has not been elucidated. Proposed functions include a role as a precursor for steroid hormones, membrane stabilization, involvement in the intrinsic coagulation system, and regulation of the skin barrier. Furthermore, it has been shown that cholesterol sulfate can block cholesterol synthesis at the level of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, and it can also block cholesterol esterification by inhibiting lecithin:cholesterol acyl-transferase.

Plasma levels of cholesterol sulfate are elevated in clinical conditions such as hypercholesterolemia and liver cirrhosis. Because cholesterol sulfate is present in the atherosclerotic lesions of human aorta and plasma levels are increased in hypercholesterolemia, cholesterol sulfate may play a role in atherosclerosis. In this study, we show that cholesterol sulfate is a substrate for platelet adhesion and may thereby contribute to the prothrombotic potential of atherosclerotic lesions.

Methods

Antibodies and Reagents

The monoclonal anti-glycoprotein Ibα antibody SZ-2 and the monoclonal anti-CD36 antibody FA6-152 were purchased from Immuno-tech. The monoclonal anti-P-selectin antibody G1 was a kind gift from Dr Rodger McEver, University of Oklahoma (Oklahoma City). F(ab’), fragments of polyclonal antibodies against von Willebrand factor and thrombospondin (Calbiochem) were prepared by pepsin digestion. All antibodies were dialyzed in HEPES-buffered saline (0.15 mol/L NaCl and 10 mmol/L HEPES, pH 7.5) before use.

Cholesterol sulfate, other sterols, and chemicals were obtained from Sigma.

Platelet Adhesion Assays

Washed platelets were prepared by centrifuging citrated blood from healthy volunteers, as previously described. They were then resuspended in HEPES-buffered saline containing 1% BSA and 1 mmol/L CaCl₂ for adhesion studies.

Wells of 96-well microtiter plates (MaxiSorp F96, Nunc) were coated with various concentrations of sterols (by evaporation of 50 μL of methanol solutions) and blocked with Tris-buffered saline (0.15 mol/L NaCl and 10 mmol/L Tris, pH 7.5) containing 5% BSA at 4°C for 2 hours. Subsequently, platelets (0.6 to 5×10⁶ cells per well) were added either unactivated in the presence of the prostacyclin analogue iloprost (0.5 μmol/L) or activated with 20 μmol/L thrombin receptor–activating peptide SFFLRNA (Ser-Phen-Phen-Leu-Arg-Asn-Ala). After incubation for 60 minutes at 37°C, nonad-
herent platelets were removed by 3 vigorous washes with Tris-buffered saline, and the samples were fixed by the addition of 4% paraformaldehyde. Bound platelets were quantified using rabbit polyclonal anti-glycoprotein Ib/IIa antibody followed by peroxidase-conjugated protein A and O-phenylenediamine as a substrate, and optical density was measured in an ELISA reader (MR 5000, Dynatech), as previously described. To determine the effect of various antibodies on the adhesion of platelets to cholesterol sulfate, platelets were preincubated with antibodies (monoclonal antibodies at 35 μg/mL and polyclonal antibodies at 100 μg/mL) for 5 minutes and added to the wells. In soluble phase-inhibition assays, platelets were preincubated with cholesterol sulfate, estrone sulfate, or vehicle control (methanol) for 5 minutes and added to the wells. The final concentration of methanol was ≈1%, and it had no effect on platelet adhesion (data not shown).

Parallel Plate Flow Chamber
The parallel plate flow chamber system included a parallel plate flow chamber, an inverted stage phase-contrast microscope (Nikon Inc, Eclipse TE300), and an imaging recording system. Glass coverslips (22×15 mm, VWR), which constitute the floor of chamber assembly, were homogeneously coated with sterols by evaporating a 100-μL methanol solution (1 mg/mL) before assembly. The chamber was maintained at 37°C by an air curtain incubator attached to the microscope.

To examine platelet adhesion to cholesterol sulfate, the chamber was perfused with citrated whole blood or platelet-rich plasma (both previously labeled with fluorescent mepacrine) at a flow rate of 8 mL/min, which generated an average wall shear stress of 10 dynes/cm² (shear rate, ≈600 s⁻¹), as previously described. Platelet adhesion was recorded through a single view field with the digital camera Quantix (Photometrics) every second for 180 seconds. The acquired data were analyzed using MetaMorph Imaging Systems software (Universal Imaging Corporation).

Results
Platelet Adhesion to Cholesterol Sulfate
We examined the adhesion of platelets to cholesterol sulfate under static conditions. Platelets adhered to surface-bound cholesterol sulfate in a concentration-dependent manner, with maximal adhesion at ≈1 to 2 μg/well cholesterol sulfate (Figure 1A). When viewed with the phase contrast microscope, the adherent platelets were spread on the surface in a single cell layer without aggregates. Platelets did not significantly adhere to cholesterol, cholesterol acetate, estrone sulfate, or dehydroepiandrosterone sulfate (Figure 1A). Soluble cholesterol sulfate inhibited the platelet adhesion to cholesterol sulfate in a concentration-dependent manner, with almost complete inhibition at 50 μg/mL, whereas soluble estrone sulfate had no effect (Figure 1B).

The adhesion of platelets to cholesterol sulfate was saturating, with maximal adhesion at ≈3.75×10⁶ platelets per microrotter well (Figure 1C). This adhesion was not divalent cation-dependent, because 10 mmol/L EDTA did not affect platelet adhesion (Figure 1C). Adhesion of platelets that were activated with 20 μM thrombin receptor–activating peptide to cholesterol sulfate was also measured, and there was an increase in adhesion after activation (Figure 1D).

The monoclonal anti-glycoprotein Ib antibody SZ-2, the chimeric anti-glycoprotein Ib/IIa antibody abciximab, the monoclonal anti-P-selectin antibody G1, the monoclonal anti-CD36 antibody FA6-152, and Fab(ab)² fragments of polyclonal antibodies against von Willebrand factor or thrombospondin did not have a significant effect (Figure 1D). However, the sulfated polyanionic glycan dextran sulfate (molecular weight, 500 000; 20 μg/mL) inhibited the interaction of platelets with cholesterol sulfate by ≈90% (Figure 1D), whereas heparin at the same concentration had only a minimal effect (data not shown), suggesting that the spatial arrangement of the sulfate group may be important for the interaction with platelets.

Platelet Adhesion to Cholesterol Sulfate Under Flow Conditions
We perfused whole blood or platelet-rich plasma over a cholesterol-coated surface in a parallel plate chamber to determine whether platelets interact with cholesterol sulfate under flow conditions. At an average shear stress of 10 dynes/cm², which is seen in arteries, there was rapid and progressive adhesion of platelets to cholesterol sulfate (Figure 2). The platelet adhesion occurred within 10 seconds and...
and that the protective effect of these statins may be mediated through changes in plaque composition rather than size. In a dyslipidemic rabbit model of atherosclerosis, platelet adhesion to damaged vessel wall placed in an ex vivo flow perfusion system was reduced in statin-treated animals compared with controls. The mechanism of decreased platelet response is not known, but it may involve a reduction in cholesterol sulfate or other thrombogenic materials in atherosclerotic plaques.

Blache et al showed that the exposure of platelets to cholesterol sulfate enhanced aggregation responses to ADP and thrombin. Thus, in addition to being a substrate for platelet adhesion, cholesterol sulfate may increase the formation of platelet aggregates.

In conclusion, cholesterol sulfate may be one of the factors determining the prothrombotic potential of atherosclerotic plaques.

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References

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