Dermatan Sulfate Inhibition of Fibrin-Rich Thrombus Formation in Nonhuman Primates

Yves Cadroy, Stephen R. Hanson, and Laurence A. Harker

Dermatan sulfate (DS), a factor that amplifies plasma heparin cofactor II antithrombin (HCII) activity, has been evaluated in baboons for its relative antithrombotic and antihemostatic effects by use of a model that combines both platelet-rich and fibrin-rich thrombus formation. Thrombus was generated in a two-component thrombogenic device incorporated into exteriorized femoral arteriovenous shunts, in which a proximal segment of collagen-coated tubing induces platelet-rich arterial-type thrombus and distal expanded chambers with disturbed and static flow produce fibrin-rich venous-type thrombus. Thrombus formation was measured as the deposition of autologous \( ^{111} \text{In}- \)platelets by imaging analysis and by the accumulation of \( ^{125} \text{I}- \)fibrin. Intravenous infusion of DS at 0.83, 8.3, and 42 mg/kg maintained plasma levels at approximately 7, 70, and 400 \( \mu \text{g/mL} \), respectively, throughout the period of study. By enhancing HCII-dependent inactivation of soluble thrombin, DS prolonged the coagulation times, reduced plasma fibrinopeptide A levels, and decreased fibrin-rich thrombus formation in the chamber portion of the device in a dose-dependent manner, ie, the intermediate dose reduced fibrin accumulation by approximately 70% \( (P<.05) \). By contrast, neither platelet deposition on collagen nor platelet hemostatic function, assessed with bleeding time determinations, was significantly affected by DS at any dose studied \( (P>.2 \) and \( P>.1, \) respectively, for the high dose), a finding presumably explained by the resistance of immobilized thrombin to inactivation by DS. (Arteriosclerosis and Thrombosis 1993;13:1213-1217)

KEY WORDS • dermatan sulfate • arterial thrombosis • venous thrombosis • heparin cofactor II • thrombin

Heparin cofactor II (HCII) inactivation of thrombin by 1:1 complex formation is potently enhanced by dermatan sulfate (DS). In rodents, DS prevents fibrin-rich venous thrombosis induced in stasis-type models. In uncontrolled studies, DS has been reported to be effective and safe in patients with venous thromboembolism. However, other antithrombin III-independent antithrombins, such as hirudin, effectively interrupt arterial thrombus formation in addition to fibrin thrombosis, although greater doses are necessary to inhibit arterial-type platelet-rich thrombus formation than venous-type fibrin-rich thrombosis. Accordingly, the efficacy of DS enhancement of HCII-mediated antithrombin activity for both arterial and venous thrombotic processes has been investigated in baboons.

Methods

Animal Studies

Eight normal male baboons (Papio anubis) weighing 8 to 12 kg were studied. The animals had been dewormed and observed to be disease-free for 3 months before use. All procedures were previously reviewed and approved by the Institutional Animal Care and Use Committee and were in compliance with procedures and methods outlined by the National Institutes of Health (Guide for the Care and Use of Laboratory Animals, National Institutes of Health, Bethesda, Md, NIH publication No. 86-23) as well as the Animal Welfare Act and related university policies.

Before studies were initiated, chronic femoral exteriorized arteriovenous (AV) access shunts were surgically placed with the animals under halothane anesthesia after induction by ketamine (10 mg/kg IM) and valium (0.5 mg/kg IV). Thereafter, ketamine hydrochloride (5 to 20 mg/kg IM) was used for subsequent short-term immobilization in experimental procedures. The shunt system provided predictable and durable blood access, thereby permitting each animal to serve as its own control. These chronic shunts do not shorten either platelet survival times or the rate of fibrinogen clearance and do not detectably activate circulating platelets or coagulation proteins.

For each experiment, a thrombogenic device was interposed in the arms of the chronic shunt system. The device consisted of a 2-cm segment of tubing (3.2-mm ID) coated with covalently bonded type I collagen, followed by two chambers of expanded diameter (each 2 cm long and 9.3-mm ID) exhibiting low-shear disturbed flow. A roller pump (Cole-Palmer, Chicago, Ill) was placed distal to the device to maintain the blood flow constant at 20 mL/min (wall shear rate in the collagen tubing calculated to be 100 second\(^{-1}\)). It has previously been shown that platelet-rich thrombus accumulates rapidly on the collagen-coated segment,
simulating arterial-type thrombosis, and that fibrin-rich thrombus forms in the chambers, simulating venous-type thrombosis.\textsuperscript{11,15}

Autologous baboon blood platelets were labeled with 1 mCi \textsuperscript{111}In-oxine according to a method described previously.\textsuperscript{11} The average labeling efficiency by this method was \textgreater 90%. After at least 1 hour was allowed for the reinforced cells to distribute within the vasculature, the thrombogenic device was incorporated into the shunt system and exposed to native nonanticoagulated blood flowing at 20 mL/min for 40 minutes. Platelet counts (420 000\(\pm\)14 000 platelets/mL), hematocrit (0.338\(\pm\)0.005), and fibrinogen levels (3.7\(\pm\)0.1 g/L) were equivalent among all study groups.

The accumulation of \textsuperscript{111}In-platelets within each component of the device was measured in real time with a gamma scintillation camera (model 4/11, Picker Corp, Northford, Conn). Data were analyzed with a computer-assisted image processing system (Medical Data Systems A\textsuperscript{2}, Medtronic, Inc, Ann Arbor, Mich) interfaced with the camera. Images were acquired at 5-minute intervals. The total number of deposited platelets (labeled plus unlabeled cells) was calculated by dividing the deposited platelet activity (counts per minute) by the whole-blood platelet \textsuperscript{111}In-activity (counts per minute per milliliter) and multiplying by the circulating platelet count (platelets per milliliter) as measured in each experiment. Radioactivity values refer to platelet activity only, with all blood measurements having been corrected for the small fraction of nonplatelet isotope in each experiment.

Homologous baboon fibrinogen was purified and labeled with \textsuperscript{125}I by the methods reported previously.\textsuperscript{11} The labeled fibrinogen preparation was \textgreater 90% clottable by thrombin. Ten minutes before thrombus formation was initiated, 5 \(\mu\)Ci of \textsuperscript{125}I-fibrinogen was administered to baboons by bolus intravenous injection. After blood exposure for 40 minutes, the device was thoroughly rinsed with isotonic saline. The collagen segment was then separated from the expanded regions, and after at least 30 days was allowed for the \textsuperscript{111}In to decay (half-life, 2.8 days), the device components were counted with a gamma counter for \textsuperscript{125}I-fibrinogen radioactivity. Total fibrin accumulation (labeled plus unlabeled) was calculated by dividing the deposited fibrin activity (counts per minute) by the clottable fibrinogen \textsuperscript{125}I-activity (counts per minute per milliliter) and multiplying by the plasma fibrinogen concentration (milligrams per milliliter) as measured in each experiment.

Bleeding times were performed in baboons on the shaved volar surface of the forearm by the standard template method.\textsuperscript{14}

### Dermatan Sulfate

DS (IC 1729, batch P 1106) was provided by Sanofi-Recherche (Gentilly, France). DS had a molecular weight of 22 kD and contained an average of one sulfate group per disaccharide unit.

To achieve a range of plasma levels, DS was administered to the animals at three different doses: 0.83 mg/kg (low dose), 8.3 mg/kg (intermediate dose), and 42 mg/kg (high dose) (see Table). Steady-state plasma levels of DS were obtained throughout the 40 minutes of study by administration of 60% of the total dose via bolus intravenous injection 3 minutes before the thrombogenic device was incorporated into the AV shunt, and the remainder was infused continuously during the subsequent 40 minutes of study.

### Laboratory Procedures

Whole blood for platelet count, hematocrit, and fibrinogen determinations was sampled before each experiment and measured as described previously.\textsuperscript{11} Systemic blood for fibrinopeptide A (FPA) measurement was obtained before and after exposure of the thrombogenic device for 40 minutes. Determinations were made by radioimmunometric assay by use of a previously reported method.\textsuperscript{11}

<table>
<thead>
<tr>
<th>Laboratory Procedures</th>
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### Effects of Dermatan Sulfate on Hemostasis

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LD</th>
<th>ID</th>
<th>HD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma level ((\mu g/mL))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 Min</td>
<td>0</td>
<td>6.7(\pm)0.6</td>
<td>75(\pm)3</td>
<td>395(\pm)67</td>
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<tr>
<td>40 Min</td>
<td>0</td>
<td>7.7(\pm)0.7</td>
<td>68(\pm)2</td>
<td>420(\pm)48</td>
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<tr>
<td>APTT (s)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>15 Min</td>
<td>32(\pm)1</td>
<td>32(\pm)2</td>
<td>60(\pm)2</td>
<td>106(\pm)7</td>
</tr>
<tr>
<td>40 Min</td>
<td>32(\pm)1</td>
<td>34(\pm)1</td>
<td>57(\pm)2</td>
<td>106(\pm)10</td>
</tr>
<tr>
<td>TCT (s)</td>
<td></td>
<td></td>
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<tr>
<td>15 Min</td>
<td>18(\pm)1</td>
<td>22(\pm)1</td>
<td>(\geq)250</td>
<td>(\geq)250</td>
</tr>
<tr>
<td>40 Min</td>
<td>18(\pm)1</td>
<td>23(\pm)1</td>
<td>(\geq)250</td>
<td>(\geq)250</td>
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<tr>
<td>FPA (pmol/L)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>40 Min</td>
<td>13.6(\pm)2.1</td>
<td>9.6(\pm)1.7</td>
<td>4.7(\pm)0.9*</td>
<td>2.9(\pm)0.3*</td>
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<tr>
<td>Bleeding time (min)</td>
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<td></td>
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</tr>
<tr>
<td>15 Min</td>
<td>3.4(\pm)0.3</td>
<td>4.7(\pm)0.8</td>
<td>5.3(\pm)0.3</td>
<td>6.2(\pm)1.2</td>
</tr>
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</table>

Baseline values before device placement were activated partial thromboplastin time, 32\(\pm\)1 sec; thrombin clotting time, 19\(\pm\)1 sec; fibrinopeptide A, 3.7\(\pm\)0.2 pmol/L. Values are mean\(\pm\)SEM of n observations. LD, low dose; ID, intermediate dose; HD, high dose. *P<.01 vs control values.
Dermatan Sulfate in Thrombus Formation

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Effect of Dermatan Sulfate on Hemostasis

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Platelet hemostatic function, as measured by the template bleeding time, was not significantly prolonged by DS; ie, at a plasma level of approximately 400 μg/mL, the bleeding time was 6.2±1.2 vs 3.4±0.3 minutes (P= .13).

Effects of Dermatan Sulfate on Thrombus Formation

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Statistical Evaluations

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Immobilized Thrombin

Effect of Dermatan Sulfate on Immobilized Thrombin

In untreated control animals (n=4), thrombin activity bound to platelet-rich thrombus formed during 30 minutes on segments of vascular grafts interposed in femoral AV shunts averaged 1.3±0.3 ng bound enzyme per segment. DS was unable to significantly decrease the activity of bound thrombin, ie, amidolytic activity of bound thrombin measured 1.1±0.3 ng bound enzyme per segment (P>.2) in the presence of 400 μg/mL DS in plasma. The effect of heparin on the catalytic activity of bound thrombin was also determined in these experiments and was found to be similarly ineffective, ie, 1.1±0.2 ng bound enzyme per segment.

Discussion

The present study shows that DS, an HCII-mediated thrombin antagonist, inhibits (1) fibrin-rich venous-type thrombus formation, (2) associated FPA production, and (3) coagulation times in a dose-related manner without affecting either platelet-rich arterial-type thrombus formation or platelet hemostatic function. The failure of DS to modify platelet thrombotic processes is explained by the finding that the combination of HCII and DS inhibits the activity of soluble thrombin but is unable to inactivate thrombin immobilized to thrombus, thereby leaving bound thrombin to mediate platelet recruitment via cleavage of the platelet-thrombin receptor.

The thrombogenic device used in this study comprises a proximal collagen-coated segment inducing platelet-rich thrombus and a distal dilated chamber of disturbed flow producing fibrin-rich thrombus.11 The relative ratio of deposited platelets to accumulated fibrin is seven times greater on the collagen segments than in the chambers.11 In the "Results," the absolute number of platelets deposited onto the collagen segments is fortuitously similar to that incorporated into the thrombus forming in the chambers. However, platelets accumulate in collagen-induced thrombus by platelet-dependent processes that are selectively antagonized by antiplatelet Arg-Gly-Asp-containing peptides,18 whereas platelets incorporate into chamber-associated thrombus by fibrin-dependent mechanisms that are inhibited by antagonists of soluble thrombin.10,11 Accordingly, the release of FPA into the circulation largely reflects the local accumulation of fibrin in the chambers of the device without significant systemic thrombin effects.

In the evaluation of the antihemostatic effects of DS, the coagulation times (APTT and TCT) were determined and found to be prolonged in a dose-dependent manner over a broad range by maintaining the plasma drug levels at approximately 7, 70, and 400 μg/mL (Table). Since no detectable anti-factor Xa activity was generated by any of these drug concentrations, it follows that DS fails to enhance antithrombin III activity or induce the release of endogenous anti-factor Xa activity.

The demonstration that DS prevents fibrin-rich venous-type thrombus formation is in accord with previous studies that used animal models of stasis venous thrombosis, ie, Wessler-type thrombosis models.3,5 In those reports, DS prevented thrombus formation at doses ranging between 150 and 500 μg/kg, corresponding to plasma concentrations of 5 to 10 μg/mL DS, levels that did not prolong either the APTT or the TCT. In the present study, the low plasma level of 7 μg/mL DS similarly failed to significantly prolong the APTT but detectably decreased fibrin-rich accumulation in the chambers of the device and reduced plasma FPA levels; ie, fibrin accumulation fell by 30% to 40% (Fig 1), and FPA levels decreased from 13.6±2.1 to 9.6±1.7 pmol/L. However, significant anticoagulant effects were seen in the coagulation tests in concert with the antithrombotic results at 70 μg/mL DS (Table). Comparable results were obtained for venous thrombosis by use of a flowing system in rabbits.19 It has previously been shown that unfractionated standard heparin or recombinant hirudin reduces fibrin-rich thrombus formation in this device by approximately 85% at 3 μg/mL (APTT, 100 seconds) and 1 μg/mL (APTT, 90 seconds), respectively.8,10

This study confirms the hemostatic safety of DS. A previous report showed that doses of DS as large as 40 mg/kg only modestly increase the blood loss from standardized ear incisions in rabbits.4 Those results correlate with our findings that 42 mg/kg of DS to baboons failed to significantly prolong the bleeding time (6.2±1.2 vs 3.4±0.3 minutes for control studies; P>.10).

At the highest dose of DS, platelet-dependent thrombus formation measured on collagen substrate was comparable to control studies with respect to both the rate and extent of platelet accumulation over the 40-minute period of blood exposure (Fig 2). This failure of DS to prevent the formation of platelet-rich thrombi on collagen segments contrasts with direct-acting AT III-independent antithrombins, such as hirudin, as evidenced by the potent antithrombotic effects of hirudin.
for platelet-dependent thrombosis in this model. Other direct antithrombins also exhibit antithrombotic efficacy for platelet-dependent arterial thrombus formation. In vitro, however, the HCII-mediated antithrombin activity produced by DS at plasma concentrations of 25 to 50 μg/mL inhibits platelet aggregation induced by thrombin. The divergent effects of inhibition of thrombin-induced platelet aggregation in vitro by DS and its lack of effectiveness for platelet-dependent processes in vivo are similar to those reported for the carboxy terminal dodecapeptide of hirudin. Since platelets are largely recruited into arterial thrombi by thrombus-bound thrombin rather than soluble thrombin, when thrombin binds with constituents in thrombus, thrombin because of prior occupancy of its thrombin-binding domain by constituents in thrombus. This interpretation is supported by the present demonstration that DS in the presence of plasma is unable to inactivate thrombin bound to thrombus (see "Results"). It is also possible that platelet factor 4 secreted from α-granules during platelet activation may antagonize HCII-mediated thrombin inhibition by DS. Although thrombin's many physiological functions are dependent on the proteolytic activity of its catalytic triad, functional specificity is determined by flanking clusters of accessory binding domains. Catalytic activation of the platelet thrombin receptor and cleavage of fibrinogen depend on substrate binding with thrombin via the anion-binding exosite of thrombin. However, when thrombin binds with constituents in thrombus, binding involves other domains shared by the heparin-antithrombin III complex. Accordingly, thrombin immobilized to thrombus is not accessible for inactivating complex formation with heparin-antithrombin III. We interpret the present data to indicate that the DS-HCII complex is similarly unable to access thrombin-bound thrombin because of prior occupancy of its thrombin-binding domain by constituents in thrombus.

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