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 $^{111}$In-platelets by imaging analysis and by the accumulation of $^{125}$I-fibrin. Intravenous infusion of DS at 0.83, 8.3, and 42 mg/kg maintained plasma levels at approximately 7, 70, and 400 µg/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).1 2 In rodents, DS prevents fibrin-rich venous thrombosis induced in stasis-type models.3-5 In uncontrolled studies, DS has been reported to be effective and safe in patients with venous thromboembolism.6 7 However, other antithrombin III-independent antithrombins, such as hirudin,6 9 effectively interrupt arterial thrombus formation in addition to venous 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.10 11

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 shunts12 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 clearance12 and do not detectably activate circulating platelets or coagulation proteins.13 14

For each experiment, a thrombogenic device was interposed in the arms of the chronic shunt system.11 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 >90%. After at least 1 hour was allowed for the reinused 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±14 000 platelets/\mu L), hematocrit (0.338±0.005), and fibrinogen levels (3.7±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{3}, 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 >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}

Plasma for measuring the activated partial thromboplastin time (APTT) and the thrombin clotting time.
(TCT) was prepared from whole blood collected in 3.8% sodium citrate (9 volumes of blood into 1 volume of citrate) 10 minutes and 40 minutes after the imaging procedure was initiated. APTT (Ortho-activated PT reagent, Ortho Diagnostic Systems, Raritan, NJ) and TCT using human thrombin (Sigma Chemical Co, St Louis, Mo) were performed with a fibrometer (Fibrosystem, Becton Dickinson, Cockeysville, Md).

The plasma concentrations of DS were determined by functional assay using the TCT with calibration curves and dilutions prepared in autologous citrated platelet-poor plasma for each animal. Thrombin catalytic activity for enzyme bound to platelet-rich thrombus was measured for thrombus formed on highly thrombogenic segments of Dacron vascular graft interposed in the arms of the exteriorized AV shunt for 30 minutes by use of a thrombin chromogenic substrate (Spectrozyme TH, American Diagnostica, Greenwich, Conn) as reported previously.16 Segments of Dacron vascular graft were used to initiate platelet-rich thrombus in these studies because thrombus remained adherent during subsequent manipulations and because the extent and composition of the platelet-rich thrombus forming on segments of vascular graft are very similar to those forming on segments of collagen-coated tubing.9,17

Thrombus-bearing segments were removed from the chronic exteriorized femoral AV shunts after 30 minutes of exposure to flowing blood and perfused with cold buffer (calcium- and magnesium-free phosphate-buffered saline) at 20 mL/min for 5 minutes. The washed thrombus-bearing segments were incubated with 200 μL of plasma for 30 minutes, followed by 30 minutes of incubation with 200 μL of a 0.25 mmol/L chromogenic substrate in 0.05 mol/L tris(hydroxymethyl)aminomethane buffer and 0.15 mol/L NaCl, pH 8.2, at room temperature. The chromogenic substrate solution was then removed and centrifuged for 2 minutes at 10 000g to remove contaminating red cells. Subsequently, the solution was filtered through a 30 000-M₅, exclusion filter (Centricon 30, Amicon Corp, Lexington, Mass) by centrifugation at 3000g for 15 minutes to remove potentially confounding hemoglobin. The supernatant (200 μL) was assayed for amidolytic product (optical density at 405 nm) and related to thrombin standards. In parallel paired preparations, one of each pair of segments was incubated with 1 μmol/L hirudin for 15 minutes before the addition of chromogenic substrate. The difference between the paired samples represented the hirudin-inhibitable activity and was defined to represent thrombin-specific amidolytic activity. The results were expressed as nanograms of thrombin catalytic activity bound to the segment.

Statistical Evaluations

Statistical evaluations were performed by use of the CLINFO programs provided by the US Department of Health and Human Services. All results are given as mean±SEM. Comparisons were made by Student's t test (two-tailed) for unpaired sample groups.

Results

Effect of Dermatan Sulfate on Hemostasis

The Table shows the effects of DS on coagulation and platelet hemostatic functions. The APTT and TCT were prolonged in a dose-response manner (APTT and TCT were minimally prolonged at 6.7±0.6 μg/mL DS and were >100 seconds at 395±67 μg/mL DS). Conversely, plasma FPA levels fell in a dose-response fashion, ie, the intermediate dose of DS abolished the fourfold increase in plasma FPA observed in control animals (4.7±0.9 pmol/L vs 3.7±0.2 pmol/L before incorporation of the thrombogenic device). No anti-factor Xa activity was detected in plasma by chromogenic assay at any of the concentrations of DS achieved (data not shown). The clotting tests were back to baseline values 24 hours later and corresponded to undetectable levels of DS in plasma.

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

DS decreased fibrin-rich thrombus formation in the chamber portion of the device in a dose-dependent manner (Fig 1). For example, the intermediate dose of DS (plasma level of about 70 μg/mL) reduced platelet deposition from 22.9±1.9×10⁶ platelets in controls to 6.3±1.1×10⁶ platelets (P=.03) and decreased fibrin accumulation from 3.3±0.4 to 1.0±0.2 mg (P=.001).

By contrast, DS failed to affect platelet-rich thrombus formation on the collagen-coated segment (Fig 2); ie, the plasma level of approximately 400 μg/mL DS was associated with platelet deposition of 17.6±4.0×10⁶ vs 22.9±1.9×10⁶ platelets in control studies (P=.30), and fibrin accumulation was 0.4±0.1 vs 0.6±0.1 mg in control experiments (P=.24).
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>0.2) 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. The relative ratio of deposited platelets to accumulated fibrin is seven times greater on the collagen segments than in the chambers. 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 anti-platelet Arg-Gly-Asp-containing peptides, whereas platelets incorporate into chamber-associated thrombus by fibrin-dependent mechanisms that are inhibited by antagonists of soluble thrombin. 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. 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. 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.

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. 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>0.10).

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.\textsuperscript{1} Other direct antithrombins also exhibit antithrombotic efficacy for platelet-dependent arterial thrombus formation.\textsuperscript{9,16,20} In vitro, however, the HClI-mediated antithrombin activity produced by DS at plasma concentrations of 25 to 50 \(\mu\)g/mL inhibits platelet aggregation induced by thrombin.\textsuperscript{15} 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.\textsuperscript{16,17,21-25} Since platelets are largely recruited into arterial thrombi by thrombus-bound thrombin rather than soluble thrombin,\textsuperscript{24,26} we postulate that steric or electrostatic hindrance prevents the large DS-HClI complexes from accessing thrombin that is immobilized to 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 \(\alpha\)-granules during platelet activation may antagonize HClI-mediated thrombin inhibition by DS.\textsuperscript{21} 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.\textsuperscript{17} Catalytic activation of the platelet thrombin receptor and cleavage of fibrinogen depend on substrate binding with thrombin via the anion-binding exosite of thrombin.\textsuperscript{24} 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.\textsuperscript{25} We interpret the present data to indicate that the DS-HClI complex is similarly unable to access thrombin-bound thrombin because of prior occupancy of its thrombin-binding domain by constituents in thrombus.

Acknowledgments

This work was supported in part by grants from the National Institutes of Health (HL-41619, HL-31649, HL-31950, and RR-00165). The authors thank Andrew B. Kelly, DVM, Deborah L. White, Ulla M. Marzec, Kristi DeBurgh, Jill Janik, and Paul McFadden for their expert technical assistance.

References

Dermatan sulfate inhibition of fibrin-rich thrombus formation in nonhuman primates.

Y Cadroy, S R Hanson and L A Harker

doi: 10.1161/01.ATV.13.8.1213

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/13/8/1213