Antithrombotic activity of dermatan sulfate in heparin cofactor II–deficient mice

Cristina P. Vicente, Li He, Mauro S. G. Pavão, and Douglas M. Tollefsen

Heparin cofactor II (HCII) is a plasma protein that inhibits thrombin rapidly in the presence of dermatan sulfate or heparin. We previously reported that the time to thrombotic occlusion of the carotid artery after photochemical injury was shorter in HCII-deficient mice than in wild-type control animals. In this paper, we describe the antithrombotic activity of dermatan sulfate in wild-type and HCII-deficient mice. Intravenous administration of porcine skin dermatan sulfate induced a dose-dependent prolongation of the carotid artery occlusion time in HCII+/+ mice that was not observed in HCII−/− mice. Pharmacokinetic studies suggested that porcine skin dermatan sulfate expresses antithrombotic activity after being transferred from the plasma to sites in the vessel wall. Using invertebrate derma
tan sulfate preparations, we showed that N-acetylgalactosamine-4-O-sulfate residues are required for the HCII-dependent antithrombotic effect. Furthermore, the invertebrate dermatan sulfates, which have higher charge densities than mammalian dermatan sulfate, slightly prolonged the thrombotic occlusion time of HCII−/− mice. These results indicate that HCII mediates the antithrombotic effect of porcine skin dermatan sulfate after injury to the carotid arterial endothelium in mice, whereas more highly charged dermatan sulfates possess weak antithrombotic activity independent of HCII. (Blood. 2004; 104:3965-3970)

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Materials and methods

Mice

HCII-deficient mice were generated by homologous recombination in 129/SvJ-derived embryonic stem cells. The resulting chimeric animals were backcrossed with inbred C57BL/6 mice purchased from Taconic (Germantown, NY). All experimental protocols were approved by the Washington University Animal Studies Committee.

Glycosaminoglycans

Dermatan sulfate from the ascidians Styela plicata and Ascidia nigra was prepared as previously described. Porcine skin dermal sulfate, porcine intestinal heparin (183 U/mg), and chondroitin 4-sulfate were purchased from Sigma (St Louis, MO). Dermatan sulfate was treated with nitrous acid to remove contaminating heparin or heparan sulfate prior to use.

Proteins

HCII was purified to homogeneity from human plasma and assayed as described previously. Murine thrombin was purchased from Haematologic Technologies (Essex Junction, VT). Antithrombin was purified to homogeneity from mouse plasma as previously described.

Arterial thrombosis model

Carotid artery thrombosis was induced as described previously. Briefly, adult male and female mice (~ 25 g body weight) were anesthetized by intraperitoneal injection of sodium pentobarbital (80 mg/kg), secured in the supine position, and placed under a dissecting microscope. The right common carotid artery was isolated through a midline cervical incision, and the occlusion time was determined as described in the preceding paragraph. Plasma (4 μL) was incubated with 125I-thrombin (0.3 nM, ~130 000 cpm) for 2 minutes at room temperature in 10 μL 0.15 M NaCl, 0.02 M Tris (tris(hydroxymethyl)aminomethane)-HCl, and 1 mg/mL polyethylene glycol, pH 7.4. The sample was then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% polyacrylamide gel under reducing conditions, and radioactive bands were detected by autoradiography. The 125I-thrombin–HCII band was cut from the gel and counted in a gamma counter.

aPTT assay

Citrin-anticoagulated mouse plasma (90 μL) was mixed with 10 μL of a glycosaminoglycan solution and 100 μL Alexa HS reagent (Sigma). After a 2-minute preincubation at 37°C, 100 μL 0.25 M CaCl2 was added and the clotting time was determined.

Thrombin inhibition assay

Glycosaminoglycans were incubated at room temperature with mouse thrombin (16 nM) and mouse antithrombin (50 nM) in 100 μL 50 mM Tris-HCl, 150 mM NaCl, 1 mg/mL poly(ethylene glycol), pH 7.4. Thrombin was added last to initiate the reaction. After 60 seconds, 500 μL 100 μM tosyl-Gly-Pro-Arg-nitroanilide (Roche Molecular Biochemicals, Indianapolis, IN) was added, and the absorbance at 405 nm was determined continuously for 100 seconds. The rate of change of absorbance was proportional to the residual thrombin activity.

Statistical analysis

Statistical significance was determined with the Student 2-tailed t test for independent samples. P < .05 was considered significant.

Results

Antithrombotic activity of porcine skin dermal sulfate in HCII+/− mice

The interval between endothelial injury and thrombotic occlusion of the carotid artery in HCII+/− animals not treated with dermal sulfate varied with their genetic background (Table 1). As previously noted, the presence of 129/SvJ alleles was associated with shorter occlusion times. Therefore, all of the experiments in this study were performed with commercial inbred C57BL/6 mice or HCII knock-out mice backcrossed for at least 10 generations into the C57BL/6 background.

Initially, we injected HCII+/− mice intravenously with 2.5 to 20 mg porcine skin dermal sulfate per kilogram body weight 15 minutes prior to the injection of rose bengal. The occlusion time increased in a dose-dependent manner, reaching a maximum of

Formation of 125I-thrombin–HCII complex ex vivo

Human thrombin was labeled with 125I as described previously. Mice were killed at various times after being injected with 10 mg/kg porcine skin dermal sulfate, and plasma was prepared as described in the preceding paragraph. Plasma (4 μL) was incubated with 125I-thrombin (0.3 nM, ~130 000 cpm) for 2 minutes at room temperature in 10 μL 0.15 M NaCl, 0.02 M Tris (tris(hydroxymethyl)aminomethane)-HCl, and 1 mg/mL polyethylene glycol, pH 7.4. The sample was then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% polyacrylamide gel under reducing conditions, and radioactive bands were detected by autoradiography. The 125I-thrombin–HCII band was cut from the gel and counted in a gamma counter.

| Table 1. Thrombosis occlusion times of untreated HCII+/− mice |
|-------------------------------|-------------------------------|-------------------------------|
| Generation | Heterozygous loci, % | Occlusion time, s, mean ± SD |
| F1 agouti | 100 | 24 ± 5 (n = 5)* |
| N6F1 | 3 | 39 ± 10 (n = 18)* |
| N10 or greater | 0.2 or lower | 59 ± 5 (n = 6) |
| Inbred C57BL/6 | 0 | 56 ± 7 (n = 9) |

F1 agouti mice were generated by mating C57BL/6 females with chimeric males derived from 129/SvJ embryonic stem cells. The F1 agouti mice were then backcrossed with inbred C57BL/6 mice. The approximate percentage of loci containing an allele from both strains (heterozygous loci) is indicated.

*Data are from He et al.
approximately twice the baseline value at a dose of 10 mg/kg (Figure 1). No further increase was observed at a dose of 20 mg/kg.

To investigate the time of onset and the duration of antithrombotic activity, we injected porcine skin dermatan sulfate 5 to 120 minutes prior to the injection of rose bengal. At a dermatan sulfate dose of 10 mg/kg, no prolongation of the occlusion time was observed when the interval between injections was only 5 minutes (Figure 2, solid circles). The occlusion time was prolonged when the interval between injections was 15 to 60 minutes but returned to baseline when injury was induced 120 minutes after injection of 10 mg/kg dermatan sulfate. In contrast, at a dose of 20 mg/kg, antithrombotic activity was observed at the 5-minute time point and persisted for the duration of the experiment (Figure 2, open circles). The occlusion time was not prolonged in control animals injected with 0.15 M NaCl 15 to 120 minutes prior to rose bengal (Figure 2, open squares).

**Pharmacokinetics of porcine skin dermatan sulfate in HCII−/− mice**

We considered several possible explanations for the delayed onset of antithrombotic activity observed at the lower dose (ie, 10 mg/kg) of dermatan sulfate: (1) dermatan sulfate injected into the tail vein could be sequestered temporarily by saturable binding sites in the pulmonary vascular bed; (2) it could be metabolized to a more active form; or (3) a portion of the dermatan sulfate could transfer from the plasma into the arterial wall in a time- and concentration-dependent manner. To investigate these possibilities, we determined the kinetics of clearance of 3H-labeled dermatan sulfate.

As shown in Figure 3A, 67% of the 3H-labeled tracer was detectable in the arterial circulation 5 minutes after injection of a dose of 10 mg/kg dermatan sulfate. The amount present in plasma decreased to 17% at 15 minutes and 7% at 60 minutes. Thus, the antithrombotic activity increased as the concentration of dermatan sulfate in plasma at the onset of endothelial injury was falling (compare Figure 2, solid circles with Figure 3A, open circles). This observation argues against temporary sequestration of dermatan sulfate in the pulmonary vasculature. An alternative explanation for the absence of an antithrombotic effect when dermatan sulfate was given 5 minutes, but not 15 minutes, before rose bengal is that rose bengal did not significantly alter the pharmacokinetics of dermatan sulfate.

To determine the activity of the circulating dermatan sulfate, plasma samples were obtained from mice at various times after injection of a 10-mg/kg dose and were incubated with 125I-thrombin in vitro. As indicated by formation of 125I-thrombin–HCII complexes, the activity of dermatan sulfate was maximal 5 minutes after injection and decreased thereafter (Figure 4). This result
suggests that dermatan sulfate was not metabolized to a more active form in the circulation. Furthermore, gel filtration and ion-exchange chromatography of plasma samples obtained 5 and 30 minutes after injection showed no alteration in the size or charge of the $^3$H-labeled dermatan sulfate (data not shown).

Approximately 55% of the injected dose of $^3$H-labeled dermatan sulfate was present in the urine after 60 minutes (Figure 3A). Despite excretion of more than half of the dermatan sulfate, antithrombotic activity was maintained when endothelial injury was initiated at this time point (compare Figure 2). Small but measurable amounts of tritium remained in the heart, lung, liver, and kidney at all time points after injection of $^3$H-labeled dermatan sulfate, even after arterial perfusion of the animal with 50 mL phosphate-buffered saline (Figure 3A-B). The amount present in heart and lung did not decrease with time in proportion to the plasma radioactivity, and the amount present in liver appeared to increase, indicating that some of the $^3$H-labeled dermatan sulfate remained in these tissues after clearance of most of the circulating material.

Antithrombotic effects of glycosaminoglycans in HCII<sup>-/-</sup> mice

We previously reported that the time to thrombotic occlusion of the carotid artery was significantly shorter in HCII<sup>+/+</sup> mice than in HCII<sup>-/-</sup> mice at both the F1 and N6F1 generation. Similarly, HCII<sup>+/-</sup> mice at the N10F1 generation, which are essentially congenic with inbred C57BL/6 animals, had significantly shorter occlusion times than their HCII<sup>+/+</sup> counterparts (37 ± 7 minutes vs 57 ± 7 minutes, $P < .0001$; Figure 5). Porcine skin dermatan sulfate (10 mg/kg) did not prolong the occlusion time of HCII<sup>-/-</sup> mice ($P = .08$; Figure 5). To confirm that prolongation of the occlusion time was HCII dependent, we administered purified HCII intravenously to HCII<sup>-/-</sup> mice to achieve a plasma concentration of approximately 0.3 mM HCII 15 minutes prior to the injection of dermatan sulfate. Reconstitution of plasma HCII resulted in prolongation of the occlusion time (88 ± 7 minutes, $n = 4$) to the same degree as that observed in HCII<sup>+/+</sup> mice ($P = .12$). In these and all subsequent experiments, the glycosaminoglycan was administered 15 minutes prior to injection of rose bengal. Heparin prolonged the occlusion time of HCII<sup>+/+</sup> mice in a dose-dependent manner (data not shown), reaching a maximum value of 97 ± 18 minutes at a dose of 0.125 mg/kg, and prolonged the occlusion time of HCII<sup>-/-</sup> mice to a similar degree ($P = .4$; Figure 5). Thus, HCII is required for the antithrombotic activity of porcine skin dermatan sulfate but plays no role in the antithrombotic activity of heparin in this experimental model.

We also determined the antithrombotic activities of dermatan sulfates isolated from 2 marine invertebrates, Stylca pilcata and Ascidia nigra. At a dose of 10 mg/kg, S plicata dermatan sulfate prolonged the occlusion time of HCII<sup>+/+</sup> mice to the same degree as did porcine skin dermatan sulfate, while A nigra dermatan sulfate was much less effective (Figure 5). The antithrombotic activity of S plicata dermatan sulfate was largely dependent on the presence of HCII, although the occlusion time of HCII<sup>-/-</sup> mice treated with S plicata dermatan sulfate was slightly longer than that of untreated HCII<sup>-/-</sup> mice (48 ± 10 minutes vs 37 ± 5 minutes, $P = .016$). Similarly, the occlusion time of HCII<sup>-/-</sup> mice treated with A nigra dermatan sulfate was slightly longer than that of untreated HCII<sup>-/-</sup> mice (53 ± 11 minutes vs 37 ± 5 minutes, $P = .002$). Increasing the dose of S plicata or A nigra dermatan sulfate to 20 mg/kg did not significantly prolong the occlusion time above that of the 10-mg/kg dose in HCII<sup>-/-</sup> mice (57 ± 4 minutes vs 48 ± 10 minutes, $P = .16$, for S plicata; and 62 ± 5 minutes vs 48 ± 10 minutes, $P = .20$, for A nigra). Thus, both of the invertebrate dermatan sulfates had weak HCII-independent antithrombotic activity in this experimental model. Chondroitin 4-sulfate did not prolong the occlusion time of HCII<sup>+/+</sup> or HCII<sup>-/-</sup> mice.

In the in vitro anticoagulant activities of the glycosaminoglycans in mouse plasma are shown in Figure 6A. S plicata dermatan sulfate
sulfate, porcine skin dermatan sulfate, and heparin prolonged the aPTT of plasma obtained from HCII\(^{+/+}\) mice, but \(A\ nigra\) dermanat sulfate did not (Figure 6A, solid symbols). In agreement with previous results obtained with human plasma, \(S\ plicata\) dermanat sulfate was about 10 times more potent than porcine skin dermanat sulfate and 10 times less potent than heparin.\(^{14}\) Neither \(A\ nigra\) nor porcine skin dermanat sulfate prolonged the aPTT of HCII\(^{−/−}\) mouse plasma, but \(S\ plicata\) dermanat sulfate was active when present at about 10 times the concentration needed to prolong the aPTT of HCII\(^{−/−}\) plasma (Figure 6A, open symbols). Heparin was equally effective in plasma from both HCII\(^{+/+}\) and HCII\(^{−/−}\) mice.

Figure 6B shows the ability of each glycosaminoglycan to stimulate inhibition of mouse thrombin by mouse antithrombin in vitro. Heparin was active at final concentrations of 0.1 \(\mu\)g/mL or higher, while porcine skin dermanat sulfate was completely inactive at the highest concentration tested (500 \(\mu\)g/mL). \(S\ plicata\) dermanat sulfate had demonstrable activity at 10 to 100 \(\mu\)g/mL, but inhibition of thrombin was incomplete under the conditions of the assay. \(A\ nigra\) dermanat sulfate had barely detectable activity at concentrations of 100 \(\mu\)g/mL or higher.

**Discussion**

Intravenous administration of porcine skin dermanat sulfate to HCII\(^{+/+}\) mice doubled the time to complete thrombotic occlusion of the carotid artery after endothelial injury but did not prolong the occlusion time of HCII\(^{−/−}\) mice (Figure 5). These results provide the first direct evidence that HCII is required for antithrombotic activity of dermanat sulfate in vivo and suggest that other potential mechanisms of action of dermanat sulfate, such as release of tissue plasminogen activator,\(^{10,11}\) are less important. Heparin inhibited thrombosis equally well in HCII\(^{+/+}\) and HCII\(^{−/−}\) mice, presumably by activation of plasma antithrombin. In these experiments, endothelial injury was induced by singlet oxygen generated from rose bengal dye (3’4’5’6’-tetrachloro-2,4,5,7-tetraiodofluorescein) in the bloodstream when exposed to green light transmitted through the vessel. Electron microscopy has shown that this procedure causes rapid detachment of the arterial endothelium, leaving the elastic lamina and underlying smooth muscle cells intact, followed by platelet and fibrin deposition.\(^{19}\) Although it is likely that HCII also mediates the antithrombotic effect of dermanat sulfate in models of stasis-induced venous thrombosis, our experiments did not address this issue.

The lowest dose of porcine skin dermanat sulfate that caused maximal prolongation of the thrombotic occlusion time in HCII\(^{+/+}\) mice was 10 mg/kg (Figure 1). Theoretically, a 10-mg/kg bolus of dermanat sulfate will produce an initial plasma concentration of 317 \(\mu\)g/mL, assuming a plasma volume of 31.5 mL/kg body weight. This concentration of porcine skin dermanat sulfate was sufficient to prolong the aPTT of plasma from HCII\(^{+/+}\) mice but did not prolong the aPTT of plasma from HCII\(^{−/−}\) mice (Figure 6). In contrast, a much lower dose of heparin (0.125 mg/kg or 23 U/kg), corresponding to an initial plasma concentration of 4 \(\mu\)g/mL (0.7 U/mL), prolonged the occlusion time of both HCII\(^{+/+}\) and HCII\(^{−/−}\) mice to the same degree. In vitro, heparin prolonged the aPTT of plasma from both HCII\(^{+/+}\) and HCII\(^{−/−}\) mice (Figure 6).

Dermatan sulfate is synthesized as a repeating polymer of \(\beta\)-glucuronic acid and N-acetyl-D-galactosamine (GalNAc), which is then modified by epimerization of \(\beta\)-glucuronic acid to \(\alpha\)-iduronic acid (IdoA), sulfation of the 2-OH group of IdoA, and sulfation of the 4- and/or 6-OH groups of GalNAc.\(^{20}\) Incomplete epimerization and O-sulfation lead to microheterogeneity of the dermanat sulfate polymers. The smallest fragment of porcine skin dermanat sulfate that bound to HCII was a hecasaccharide composed of 3 Ido 2-sulfate–GalNAc 4-sulfate disaccharides.\(^{13}\) The disaccharide subunit of the HCII-binding hecasaccharide is a minor component of porcine skin dermanat sulfate, comprising only 5% of the total disaccharides. Although this hecasaccharide increased the rate of thrombin inhibition approximately 100-fold, dermanat sulfate chains 12 to 14 residues in length were necessary for maximal stimulation of HCII (\(\sim 10\,000\)-fold).\(^{21,22}\) In the current study, we used unfractionated porcine skin dermanat sulfate in which the majority of chains contain more than 14 residues.

We obtained information about the structural features of dermanat sulfate required for antithrombotic activity by comparing mammalian and invertebrate preparations of known composition. The dermanat sulfates isolated from \(S\ plicata\) and \(A\ nigra\) both have a higher content of IdoA 2-sulfate residues (66%-80%) than do typical mammalian dermanat sulfates (5%-7%), and they differ in the pattern of sulfation of GalNAc.\(^{14}\) The GalNAc residues of \(S\ plicata\) dermanat sulfate are 94% 4-sulfated and 6% 6-sulfated, whereas those of \(A\ nigra\) are 100% 6-sulfated. Despite comparable overall degrees of sulfation, \(A\ nigra\) dermanat sulfate stimulated thrombin inhibition by purified HCII only at very high concentrations, whereas \(S\ plicata\) dermanat sulfate was 1000 times more active.\(^{14}\) This observation confirmed the importance of GalNAc 4-sulfate residues for stimulation of HCII. However, the presence of GalNAc 4-sulfate was not sufficient for stimulation of HCII, because the repeating polymer of glucuronic acid and GalNAc 4-sulfate (ie, chondroitin 4-sulfate) was inactive.\(^{1}\) In comparison with mammalian dermanat sulfate, \(S\ plicata\) dermanat sulfate contains approximately 10 times more IdoA 2-sulfate and was 10
times more active with purified HCII. Thus, there is a good correlation between the abundance of residues found in the HCII-binding hexasaccharide (IdoA 2-sulfate and GalNAc 4-sulfate) and the ability of dermran sulfate both to stimulate HCII in vitro,

Although the anticoagulant activity of S plicata dermran sulfate in vitro was approximately 10 times greater than that of porcine skin dermran sulfate (Figure 6),

In vitro1,5,14 and to produce an antithrombotic effect in vivo (Figure 5).

These results suggest that there may be important differences in the pharmacokinetics and/or pharmacodynamics of porcine skin and S plicata dermran sulfate. Moreover, S plicata dermran sulfate had substantial activity in the aPTT assay with plasma from HCII+/− mice and caused a small but significant prolongation of the thrombotic occlusion time in HCII+/− mice. A nigra dermran sulfate also prolonged the occlusion time slightly in HCII+/− mice but had no activity in the aPTT assay. Although neither S plicata nor A nigra dermran sulfate stimulated inhibition of factor Xa or thrombin by human antithrombin,14 we found that both of the invertebrate dermran sulfates weakly stimulated inhibition of mouse thrombin by mouse antithrombin (Figure 6B). Therefore, stimulation of the thrombin-antithrombin reaction could contribute to the weak antithrombotic activity of these glycosaminoglycans in HCII+/− mice.

The pharmacokinetic behavior of dermran sulfate in HCII+/− mice is complex and remains incompletely understood. We observed that the antithrombotic activity of dermran sulfate did not correspond to the amount or activity of the glycosaminoglycan present in plasma (Figures 2–4) at the onset of endothelial injury. The amount of 3H-labeled dermran sulfate recovered in plasma and urine during the interval from 15 to 60 minutes was substantially less (40%-60%) than the total amount injected (Figure 3).

Examination of saline-perfused heart, lung, and liver indicated that a small percentage of the administered dermran sulfate (~4%) was detectable in these organs 60 minutes after injection. Presumably, the remainder of the dermran sulfate was sequestered in other tissues. Similar experiments demonstrating rapid plasma clearance, urinary excretion, and deposition of radiolabeled dermran sulfate in tissues have been performed in rabbits and rats.12,13 These observations suggest that porcine skin dermran sulfate expresses antithrombotic activity after being transferred from the plasma to sites in the vessel wall that remain to be identified. The relatively low specific radioactivity that we were able to achieve by tritium labeling of dermran sulfate (~10 000 dpm per microgram) precluded detection in an isolated arterial segment, whose mass is miniscule in comparison with that of the heart, lung, or liver.

In conclusion, we have demonstrated that porcine skin dermran sulfate administered intravenously to mice inhibits thrombosis of the carotid artery after endothelial injury and that the antithrombotic effect requires HCII. By contrast, the antithrombotic effect of S plicata dermran sulfate depends mainly, but not entirely, on the presence of HCII; the weak HCII-independent activity of S plicata and A nigra dermran sulfate in the thrombosis model may be related to their much greater charge density in comparison with porcine skin dermran sulfate. It remains to be determined whether dermran sulfate molecules present endogenously in the vessel wall also stimulate HCII to inhibit thrombus formation.

References

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