Fibrinogen Inhibits the Heparin Cofactor II–Mediated Antithrombin Activity of Dermatan Sulfate

By Adrian Zammit and Joan Dawes

Dermatan sulfate is a naturally occurring antithrombotic glycosaminoglycan consisting of alternating residues of N-acetylgalactosamine and iduronic acid. It is able to inhibit coagulation through interaction with heparin cofactor II (HCII), which potentiates the inactivation of thrombin and reduces thrombin generation in plasma. However, because of its inability to interact with antithrombin (ATIII), dermatan sulfate has a much lower anticoagulant activity than standard heparin on a gravimetric basis.

In experiments with animal models as well as in recent clinical trials, dermatan sulfate has been shown to be an effective agent in the prophylaxis and treatment of venous thrombosis, with a much lower hemorrhagic risk than unfractionated and low molecular weight (LMW) heparins. In addition, dermatan sulfate appears to be a more potent inhibitor of fibrin accleration onto existing thrombi than heparin when administered at equivalent antithrombin activities, suggesting that in some clinical situations dermatan sulfate may be a better antithrombotic agent than heparin. However, the mechanism by which dermatan sulfate displays its antithrombotic activities in vivo still remains to be clarified.

The antithrombotic activity of dermatan sulfate observed clinically as well as in animal models was not clearly related to its anticoagulant activity as measured by several in vitro assays in some studies, but there was a correlation with prolongation of the thrombin clotting time in recent work where higher levels of dermatan sulfate were generated.

Another property of dermatan sulfate that may contribute to its value as an antithrombogenic drug is its apparent resistance to neutralization by nonanticoagulant plasma proteins. Histidine-rich glycoprotein, vitronectin, and platelet factor 4 have all been shown to modulate the anticoagulant activity of standard and LMW heparins, but appear to be much less effective in neutralizing dermatan sulfate.

Indeed, Cosmi et al have recently reported that in contrast with standard heparin, the in vitro antithrombin activity of dermatan sulfate measured in plasma from heparin-resistant patients is equal to that in plasma from healthy volunteers.

In this investigation, we show that in normal human plasma the HCII-mediated antithrombin activity of dermatan sulfate is inhibited by approximately twofold compared with its activity in buffer and show that fibrinogen modulates this activity in a concentration-dependent manner.

MATERIALS AND METHODS

Materials. All dermatan sulfate preparations used in this investigation were supplied by Mediolanum farmaceutici SpA (Milan, Italy). These were (1) an unfractionated dermatan sulfate from porcine intestinal mucosa (MF701, batch 48), (2) MF701-derived dermatan sulfate fractions prepared by ion-exchange chromatography displaying low (low-affinity; 701R06A) and high (high-affinity; 701R06B) in vitro HCII-mediated antithrombin activity, (3) a slightly oversulfated dermatan sulfate (low-affinity oversulfated; 701R06A/S) derived from 701R06A and with a similar activity to 701R06B, and (4) a LMW (7083) dermatan sulfate. Some of their properties are listed in Table 1. A monoclonal antibody to human apolipoprotein E was purchased from Boehringer Mannheim Australia (Sydney); purified human apolipoprotein E, from Cappel, Organon Teknika NV (Leuven, Belgium); human thrombin and serum albumin, from Sigma (St. Louis, MO); the chromogenic substrate S2238 for thrombin from Chromogenix (Molndal, Sweden); and cyanogen bromide (CNBr)-activated Sepharose 4B from Pharmacia Australia (Sydney). Purified human HCII, histidine-rich glycoprotein, and fibrinogen were purchased from Servo (Gennevilliers, France). Recombinant y(Tyr63)-hirudin CPG 39393, produced in collaboration with Plantorgan Werk KG, was a gift of Ciba-Geigy, Basel, Switzerland. All other reagents, purified proteins, and antiserum, as well as human plasma prepared from hirudin- or citrate-anticoagulated pooled blood, were as described previously.

Measurement of HCII-mediated antithrombin activity catalyzed by dermatan sulfate in plasma and phosphate-buffered saline. The antithrombin activity of the various dermatan sulfate preparations in plasma and phosphate-buffered saline (PBS; 20 mmol/L sodium phosphate buffer, pH 7.4, 0.15 mol/L NaCl) was measured at 25°C using the chromogenic substrate S2238 as described previously, with slight modifications. Briefly, an appropriate amount of dermatan sulfate in citrate anticoagulated human plasma diluted 5- to 20-fold in PBS or in PBS containing a concentration of purified human HCII equivalent to that found in the diluted plasma was incubated for 5 minutes at 25°C. Human thrombin (final concentration, 0.4 U/mL; 3.7 mmol/L) also equilibrated at 25°C was then added to give a final volume of 0.25 mL and quickly mixed, and at 10-second intervals...
Table 1. Properties of Dermatan Sulfates MF701, 701RO6A, 701RO6B, and 701RO6A/S and the LMW Dermatan Sulfate 7083

<table>
<thead>
<tr>
<th></th>
<th>MF701</th>
<th>701RO6A</th>
<th>701RO6B</th>
<th>701RO6A/S</th>
<th>7083</th>
</tr>
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<tbody>
<tr>
<td>Molecular weight (D)</td>
<td>26,800</td>
<td>18,600</td>
<td>20,200</td>
<td>19,000</td>
<td>7,500</td>
</tr>
<tr>
<td>Organic sulfur (%)</td>
<td>6.67</td>
<td>6.23</td>
<td>6.51</td>
<td>7.60</td>
<td>6.75</td>
</tr>
<tr>
<td>Antithrombin activity:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC 50% via HCII (µg/mL)</td>
<td>4.0*</td>
<td>2.0</td>
<td>0.28</td>
<td>0.34</td>
<td>0.64</td>
</tr>
</tbody>
</table>

* MF701 batch 48 had a lower in vitro antithrombin specific activity than that used for the preparation of 701RO6A, 701RO6B, 701RO6A/S, and 7083 dermatan sulfate fractions.

30-µL aliquots were added to a 96-well microtiter plate containing 0.2 mmol/L S2238 in 135 µL 50 mmol/L Tris HCl (pH 8.4), 150 mmol/L NaCl, 0.1% (vol/vol) Tween-20, 0.25% (wt/vol) gelatin, and 50 µg/mL polybrene. After the amidolytic activity was allowed to proceed at room temperature for 15 minutes, the reaction was terminated by the addition of 90 µL 20% (vol/vol) acetic acid, and the optical density at 405 nm was measured at various time points (V) was measured in a Titertek Multiskan MCC1340 plate reader (ICN Biomedicals, Sydney, Australia). For the measurement of thrombin amidolytic activity at time zero (V₀), the assays were performed as indicated above in the absence of HCII. The kinetic analysis of thrombin inhibition was performed in citrate anticoagulated human plasma diluted 1:5 in PBS, or in PBS containing a concentration of purified HCII equivalent to that found in this concentration of plasma (0.25 µmol/L). In these experiments the concentration of HCII and thrombin was fixed at 250 and 3.7 nmol/L, respectively, and the dermatan sulfate concentration was varied as indicated in the text. Pseudo-first-order rate constants were obtained by plotting the semilog of residual thrombin activity (V/V₀) against time. Under these assay conditions, thrombin inhibition followed pseudo-first-order kinetics for at least 40 seconds.

Immuno depletion of HCII from human plasma. Whole rabbit antiserum containing monospecific polyclonal antibodies to human HCII (0.3 mL) was coupled with CNBr-activated sepharose 4B (0.86 g) according to the manufacturer’s instructions. Briefly, 0.3 mL HCII antiserum diluted in 6 mL of 0.1 mol/L NaHCO₃ (pH 8.3), 0.5 mol/L NaCl (coupling buffer) was added to the activated sepharose 4B and rotated for 2 hours. Excess antiserum was removed by washing with 30 mL coupling buffer, and excess reactive groups on the gel were blocked by mixing in 6 mL of 0.1 mol/L Tris/HCl (pH 8) for 2 hours. The immobilized HCII antiserum was finally washed three times with 20 mL of 0.1 mol/L acetate buffer (pH 4), 0.5 mol/L NaCl, 20 mL of 0.1 mol/L Tris HCl (pH 8), and 0.5 mol/L NaCl, and once with 100 mL PBS. All of the above steps were performed at room temperature.

Human HCII-depleted plasma was prepared by adding the immobilized HCII antiserum to 0.3 mL of normal human citrated plasma diluted to a final volume of 1 mL in PBS. The slurry was gently rotated end-over-end overnight at 4°C, and the plasma was collected after centrifugation at 1,000g for 2 minutes. Densitometric analysis of a Western blot indicated that approximately 92% of HCII was removed from the plasma sample (not shown).

Immobilization of dermatan sulfate. Coupling of dermatan sulfate MF701 to divinylsulphone-activated agarose and quantitation of dermatan sulfate immobilized on agarose were performed as described previously. Immobilized sulfate groups were quantitated by toluidine blue titration and converted to milligrams per milliliter of immobilized dermatan sulfate using the organic sulfur content of the preparation (Table 1). Approximately 0.49 mg of MF701 was covalently linked to 1 mL of agarose.

Binding of plasma proteins to immobilized dermatan sulfate. Binding of proteins to immobilized dermatan sulfate in whole human plasma prepared from hirudin-anticoagulated pooled blood was performed as described previously. Briefly, 0.1 mL of 10% (wt/vol) agarose, to which dermatan sulfate had been covalently complexed, was incubated with 1 mL of human plasma at room temperature for 30 minutes. Protein bound to the immobilized dermatan sulfate was separated from unbound protein by sedimentation under gravity for 20 minutes through 1 mL of 50 mmol/L sodium phosphate (pH 7.4), containing 1% (vol/vol) Tween-20 and 10% (wt/vol) sucrose. After washing, protein bound to the immobilized dermatan sulfate gels was eluted with 0.1 mL of 50 mmol/L sodium phosphate (pH 7.4) containing 2 mol/L NaCl and stored at −20°C.

Analysis and quantitation of proteins bound to immobilized dermatan sulfate. Proteins bound to immobilized dermatan sulfate...
Fig 2. Double-reciprocal plots of the rate of HCII-mediated thrombin inactivation catalyzed by varying concentrations of unfractionated (A; MF701), low-affinity (B; 701RO6A), high-affinity (C; 701RO6B), low-affinity oversulfated (D; 701RO6A/S), and LMW (E; 7083) dermatan sulfate in purified systems and in whole human plasma. Rates of thrombin (3.7 nmol/L) inactivation in the presence of varying concentrations of dermatan sulfate were measured under pseudo-first-order conditions in normal plasma diluted 1:5 in PBS or in PBS containing a fixed concentration of purified HCII (250 nmol/L; ○). Residual thrombin activity was measured using a chromogenic substrate as described in Materials and Methods. The apparent first-order rate constant of the reaction (\(\Delta k'\)) was obtained from the initial slope of a semilog plot of thrombin activity against time.

were analyzed in reducing sodium dodecyl sulfate (SDS)-polyacrylamide gels, identified by Western blotting, and quantitated by slot blotting and Western blotting as described previously. For each quantitation, standard curves were prepared using serial dilutions of pooled human plasma that had been previously standardized with purified proteins of known concentration to ensure that the response was linear within the range of the experiment.

RESULTS

Antithrombin activities of the dermatan sulfate preparations in plasma and in purified systems. The antithrombin activity of 0.25 \(\mu\)mol/L high-affinity (701RO6B) dermatan sulfate in human plasma was ~55% of that measured in PBS containing purified HCII at a concentration equivalent to that found in plasma (Fig 1). A similarly low antithrombin activity was observed in HCII-depleted human plasma to which purified HCII at a concentration equivalent to that in normal plasma was added (Fig 1). When the activities of the other dermatan sulfate preparations were measured in normal human plasma, they were also ~55% of those measured in purified systems (data not shown). The degree to which the antithrombin activity of all the dermatan sulfate preparations was inhibited in plasma compared with that in PBS remained unchanged when assayed in normal human plasma diluted 5- to 20-fold (data not shown), and all experiments were performed under these conditions.

The kinetics of inactivation of human thrombin by HCII in whole human plasma and in purified systems were examined under pseudo-first-order conditions as a function of the concentration of dermatan sulfate. All double-reciprocal plots of pseudo-first-order rate constants (\(\Delta k'\)) as a function of the concentration of the various dermatan sulfate preparations were linear, with plots of data obtained in plasma and in PBS intersecting to the left of the ordinate (Fig 2). The values for the maximum first-order rate constant (\(\Delta k'_{\text{max}}\)) and apparent dissociation constant (\(k_d\)) obtained by analysis of the data shown in Fig 2 are given in Table 2.

Identification and quantitation of proteins bound to immobilized dermatan sulfate. To identify the protein(s) potentially responsible for the modulation of dermatan sulfate antithrombin activity in human plasma, the profile of proteins bound to immobilized unfractionated dermatan sulfate (MF701) was analyzed. When 0.1 mL of the MF701-agarose preparation (containing 2.0 nmol of covalently linked MF701) was incubated in 1 mL whole hirudin-anticoagulated human plasma and the bound proteins were analyzed
Table 2. Kinetic Constants for the HCII-Mediated Inhibition of Thrombin Catalyzed by Unfractionated (MF701), Low-Affinity (701R06A), High-Affinity (701R06B), Low-Affinity Oversulfated (701R06A/S); and LMW (7083) Dermatan Sulfate in Human Plasma and in Purified Systems

<table>
<thead>
<tr>
<th>Dermatan Sulfate</th>
<th>Δk'_{max} (min⁻¹)</th>
<th>k_d (µmol/L)</th>
<th>Δk'_{max} (×10⁻⁶ mol/L⁻¹/min⁻¹)</th>
<th>Δk'_{max}/k_d (min⁻¹)/µmol/L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>PBS</td>
<td>Plasma</td>
<td>PBS</td>
</tr>
<tr>
<td>Unfractionated</td>
<td>6.5</td>
<td>31.8</td>
<td>0.85</td>
<td>2.7</td>
</tr>
<tr>
<td>Low-affinity</td>
<td>3.2</td>
<td>7.8</td>
<td>0.88</td>
<td>1.4</td>
</tr>
<tr>
<td>High-affinity</td>
<td>4.4</td>
<td>13.0</td>
<td>0.31</td>
<td>0.51</td>
</tr>
<tr>
<td>Low-affinity/OS</td>
<td>3.9</td>
<td>13.6</td>
<td>0.33</td>
<td>0.75</td>
</tr>
<tr>
<td>LMW</td>
<td>3.5</td>
<td>23.0</td>
<td>0.73</td>
<td>4.1</td>
</tr>
</tbody>
</table>

The maximum pseudo-first-order rate constant (Δk'_{max}) and the apparent dissociation constant (k_d) were obtained from the intercepts on the ordinate and abscissa, respectively, of the double reciprocal plots shown in Fig 2. The slope of the double-reciprocal plots is given by Δk'_{max}/k_d, and the second-order rate constant (Δk'_{max}) was calculated by dividing Δk'_{max} by the concentration of HCII in the reaction.

Abbreviation: OS, oversulfated.

in a silver-stained 10% reducing SDS-polyacrylamide gel, 10 to 12 polypeptide bands were observed (Fig 3). A similar number of bands, albeit at a reduced intensity, were also evident in the lane containing proteins bound to the control agarose. The major bands were identified and quantitated by Western blotting and slot blotting as HCII, histidine-rich glycoprotein, apolipoprotein E, fibrinogen, vitronectin, fibrinectin, and human serum albumin. The amounts specifically bound are given in Table 3. In each case, the amount of a protein nonspecifically bound to 0.1 mL ethanolamine-blocked agarose (ie, 89 ng histidine-rich glycoprotein, 57 ng HCII, 960 ng fibrinogen, 200 ng vitronectin, and 75 ng fibrinectin) was subtracted from that bound to 0.1 mL immobilized dermatan sulfate; binding of serum albumin was completely nonspecific. On a molar basis, histidine-rich glycoprotein and apolipoprotein E were the most abundant proteins bound, together with significant amounts of fibrinogen and vitronectin. The binding profile was unchanged when citrate was used as anticoagulant, and the same proteins bound to the high-affinity dermatan sulfate preparation 701R06B (data not shown).

Identification of protein(s) responsible for the inhibition of
dermatan sulfate. The activity of 0.25 μmol/L high affinity (701R06B) dermatan sulfate was assayed in purified systems in the presence of those proteins that specifically bound to immobilized dermatan sulfate in whole human plasma. In an assay system in which all purified proteins (except for thrombin) were added at concentrations equivalent to that found in plasma diluted 15-fold, histidine-rich glycoprotein, vitronectin, apolipoprotein E, and serum albumin had no effect on the HCII-mediated antithrombin activity of dermatan sulfate, whereas 0.59 μmol/L purified fibrinogen (equivalent to a plasma concentration of 3 mg/mL) inhibited the dermatan sulfate-catalyzed reaction by 30.1% ± 3% (n = 3; Fig 4). Identical results were obtained with the other dermatan sulfate preparations (data not shown).

The degree to which purified human fibrinogen inhibited the antithrombin activity of high-affinity (701R06B) dermatan sulfate was dependent on its concentration (Fig 5). When the concentration of fibrinogen was varied from 0.2 to 0.9 μmol/L, the degree of inhibition increased from 16% to 46%, reaching a maximum inhibition of ~50% at fibrinogen concentrations ~1 μmol/L. The range of fibrinogen levels used in the assays (0.2 to 1.4 μmol/L) represents a concentration of 1 to 7 mg/mL in whole human plasma.

**DISCUSSION**

In this investigation we show for the first time that the HCII-mediated antithrombin activity of dermatan sulfate was reduced in whole human plasma by approximately twofold in comparison with that measured in purified systems and that fibrinogen is probably responsible for this inhibition. Experiments with HCII-depleted plasma reconstituted with purified HCII confirmed that this effect was a bona fide inhibition caused by a plasma protein(s) and not caused by differences in activity between the purified HCII used for assays in buffer and that of the endogenous protein in
plasma. In addition, our results show that fibrinogen at a concentration equivalent to that found in normal plasma (3 mg/mL) inhibited the antithrombin activity of a high-affinity dermatan sulfate (701R06B) by approximately 30%, whereas other proteins that bound to immobilized dermatan sulfate, namely histidine-rich glycoprotein, apolipoprotein E, and vitronectin, had no effect. Similarly fibrinogen-fibrin, has been reported not to inhibit the HCII-mediated inhibition of thrombin catalyzed by dermatan sulfate,26 a high concentration of thrombin was used in that study and the investigators specifically comment that they did not study inhibition of 2 to 20 nmol/L thrombin; thus, there is no real discrepancy with the results reported here, which pertain to a lower, more physiologically relevant concentration. The inability of physiologic concentrations of histidine-rich glycoprotein and vitronectin to modulate the HCII-mediated inactivation of thrombin by dermatan sulfate are in accordance with previous reports.20,21,29 The apparent discrepancy in the degree of inhibition of the antithrombin activity of dermatan sulfate in whole plasma (45% inhibition) and that caused by purified fibrinogen (30% inhibition) in our experiments may be a result of partial denaturation of the purified protein, although the possibility that additional proteins may also be involved in the inhibition of the dermatan sulfate antithrombin activity in plasma cannot be excluded.

Kinetic analysis of the dermatan sulfate-catalyzed inactivation of thrombin indicated that the reduction in the rate of reaction in plasma was due to noncompetitive inhibition with respect to dermatan sulfate. This suggests that the inhibitor(s) in plasma affected the rate of thrombin inactivation catalyzed by dermatan sulfate not by directly competing with HCII and thrombin for binding with the glycosaminoglycan, but by modulating the rate of formation of the thrombin-HCII complex. As thrombin binds to both fibrinogen30 and dermatan sulfate1,12 at sites distinct from the protease catalytic center,20,32 our results are consistent with the possible formation of a quaternary fibrinogen-thrombin-HCII-dermatan sulphate complex, resulting in the protection of thrombin from inactivation by HCII. Our observations that fibrinogen inhibited the various dermatan sulfate fractions to exactly the same extent, irrespective of their degree of sulfation and molecular size, adds further support to an inhibitory mechanism for fibrinogen that does not involve direct competition with HCII and thrombin for binding to dermatan sulfate. Indeed, such a mechanism has been previously proposed for the inhibitory effect of fibrin(ogen) on the heparin-catalyzed inactivation of thrombin via ATIII.26,27 It should be noted that our results cannot eliminate the possibility that the inhibitory effect observed is not caused by fibrinogen per se, but by fibrin locally formed on the dermatan sulfate by thrombin.

The catalytic efficiency of the various dermatan sulfate preparations in plasma and in PBS, as measured by $\Delta K_{\text{cat}}/K_{\text{d}}$ (Table 2), was closely correlated with the in vitro antithrombin activities supplied by the manufacturer (Table 1), although this correlation did not extend to unfractionated MF701. The fractions were prepared from a different and more potent batch of MF701 than the unfractionated material tested (Mediolanum farmaceutici, personal communication, April 1994), and differences between batches may account for this discrepancy. As a result of the noncompetitive inhibition, the kinetic parameters of the dermatan sulfate-catalyzed reaction obtained in plasma are significantly different from those obtained in purified systems, with differences between the kinetic parameters for the various dermatan sulfate fractions in plasma being much smaller than those obtained in PBS (Table 2). For example, the difference between the highest and lowest apparent dissociation constants (kd) in PBS, obtained for the LMW and high-affinity dermatan sulfate fractions, respectively, was eightfold compared with 2.3-fold in plasma. Similarly, the difference between the highest and lowest second-order rate constants ($\Delta K_{\text{cat}}$) in purified systems, obtained for the unfractionated and low-affinity dermatan sulfates, respectively, was 4-1-fold compared with twofold in plasma (Table 2). This suggests that the anti-thrombin properties of dermatan sulfate preparations measured in purified systems may not accurately reflect those displayed in whole human plasma, and may explain at least in part why an oversulfated dermatan sulfate preparation that shows a greatly enhanced catalytic activity in vitro does not display any improved antithrombotic properties in animal models.33

The current study suggests that the dose response of clinically administered dermatan sulfate may display interindividual variability, depending on the patient’s plasma fibrinogen concentration. The apparent contrast of our data with those of Cosmi et al22 results from their use of defibrinated plasma. Although we found that inhibition of the antithrombin activity of dermatan sulfate varied over the normal range of 2 to 4.5 mg/mL,34 effects may be particularly severe in patients with the acute-phase reaction of thrombotic disease, where plasma fibrinogen levels can exceed 7.5 mg/mL.35 However, further work is required to assess the clinical implications of plasma fibrinogen levels on the therapeutic efficacy of dermatan sulfate. In addition, our observation that the anticoagulant activity of dermatan sulfate may be modulated in human plasma by fibrinogen may, in part at least, explain why elevated plasma levels of fibrinogen are associated with an increased risk of myocardial infarction, stroke, and atherosclerosis.34

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Fibrinogen inhibits the heparin cofactor II-mediated antithrombin activity of dermatan sulfate

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