The Relative Importance of Thrombin Inhibition and Factor Xa Inhibition to the Antithrombotic Effects of Heparin

By M. R. Buchanan, B. Boneu, F. Ofosu, and J. Hirsh

The relative importance of antithrombin and anti-factor Xa activities of heparin fractions required to achieve optimal antithrombotic effects is unknown. To study this, we measured the effects of standard heparin, an octasaccharide heparin fraction (anti-factor Xa activity only), and dermatan sulfate (antithrombin activity only) on the prevention of thrombosis and related this to their anticoagulant effects in vivo in rabbits. Thrombosis was measured as the incorporation of $^{125}I$-fibrinogen into tissue thromboplastin-induced thrombi using a Wessler-type model. Ex vivo changes in thrombin clotting time (TCT) were used as an index of antithrombin activity, and a chromogenic anti-factor Xa assay was used to measure anti-factor Xa activity. In addition, the ability of the three sulfated polysaccharides to simultaneously inhibit the generation of thrombin activity and to enhance the inactivation of the factor Xa added to initiate thrombin generation in plasma was determined. Standard heparin, in a dose of 10 anti-factor Xa U/kg, inhibited thrombus formation by 90%, prolonged the TCT by two seconds, and resulted in an anti-factor Xa level of 0.32 U/mL. The octasaccharide heparin fraction, in a dose of 10 anti-factor Xa U/kg, inhibited thrombus formation by 41%, had no effect on the TCT, and resulted in an anti-factor Xa level of 0.28 U/mL. Higher doses of the octasaccharide resulted in a further increase in the anti-factor Xa levels but had no further effect on thrombus formation. Dermatan sulfate, in a dose of 500 μg/kg, inhibited thrombus formation by 95%, but had no effect on the TCT. These results indicate that the antithrombotic effect achieved by inhibiting factor Xa is limited and that better antithrombotic effects are achieved by heparin or heparin-like substances capable of influencing the inactivation and/or the generation of thrombin.

R ECENT OBSERVATIONS in experimental animals suggest that some low molecular weight fragments of heparin have antithrombotic effects in animals with significantly reduced risk of hemorrhagic complications. Low molecular weight heparins generally have higher anti-factor Xa activity than antithrombin activity in plasma, and it has been suggested that the higher anti-factor Xa activity is responsible for the antithrombotic effects of low molecular weight heparins. However, we and others have shown that derivatives of heparin with very high ratios of anti-factor Xa to antithrombin activity are poor antithrombotic agents in experimental animals.

The present study was performed in an attempt to determine the relative importance of the antithrombin and anti-factor Xa activities of heparin to the antithrombotic properties of heparin. The following sulfated polysaccharides were evaluated: an octasaccharide heparin derivative with anti-factor Xa activity only; dermatan sulfate with antithrombin activity only; and standard heparin with equivalent anti-factor Xa to antithrombin activity. We also investigated the ability of the three agents to inhibit the generation of thrombin activity in plasma and to enhance the inactivation of the factor Xa added to initiate thrombin generation. Our results indicate that heparin fractions with predominantly anti-factor Xa activities are likely to be weak inhibitors of thrombin generation. Conversely, the ability of heparin or heparinoid to potentiate the inactivation or the generation of thrombin appears to be the important predictor of the antithrombotic effect observable in vivo.

MATERIALS AND METHODS

Porcine mucosal heparin (specific activity 150 U/mg both in USP and anti-factor Xa units) was obtained from Diosynth BV (Oss, The Netherlands). An octasaccharide (BC7-19B) derived from heparin (manufacturer’s specific activity 0 USP U/mg and 500 anti-factor Xa U/mg) was obtained from Choay Pharmaceuticals (Paris). Automated activated partial thromboplastin time (APTT) reagent was obtained from General Diagnostics (Toronto). Human brain cephalin was prepared by the method of Bell and Alton. Fatty acid-free bovine serum albumin, porcine skin dermatan sulfate (lot number 109C-2314), and other reagent-grade chemicals were obtained from Sigma Chemical Co (St Louis). The chromogenic substrates S-2222 (CBz-Ile-Gly(OR)-Gly-Arg-pNA-HCl) and S-2238 (H-D-Phe-Pip-Arg-pNA) were purchased from Pharmacia (Dorval, Quebec, Canada). $^{125}I$ NaI (610 μCi/mL carrier-free) was obtained from New England Nuclear (Boston). Sodium pentobarbital was obtained from MTC Pharmaceuticals (Hamilton, Ontario, Canada). Tissue thromboplastin was obtained from the National Reference Laboratory, Worthington Hospital, Manchester, England. The activated PTT reagent used in the APTT assays was Dade Actin-PS reagent, lot SA-13A and SA-18B. Fibrinogen was prepared from rabbit pooled plasma and iodinated according to the methods of Regoecci.

Coagulation Assays

The effects of heparin on coagulation in vitro and ex vivo were measured by the APTT and TCT assays according to standard methods and by a chromogenic anti-factor Xa assay as described by Teien et al. Standard curves for each assay were generated using homologous pooled rabbit plasma.
Experimental Thrombosis Model

Tissue thromboplastin-induced, Wessler-type thrombi were produced in jugular veins of rabbits according to standard methods described by us. Briefly, the rabbits were anesthetized with sodium pentobarbital (30 mg/kg) given intraperitoneally, and each jugular vein was isolated between two loose sutures. The rabbits were injected with 100 μg of 125I-fibrinogen and then with the heparin being tested (standard heparin, octasaccharide, or dermatan sulfate). Five minutes later, 1,000 μg/kg of tissue thromboplastin was injected via the carotid artery over ten seconds. Ninety seconds later, both jugular vein segments were occluded by the distal and proximal sutures, and opened longitudinally 30 minutes later. The size of the formed thrombus within each isolated vessel wall segment was evaluated both by visual scoring and radioactive determinations.

Data Handling and Analysis

All data were analyzed using one- and two-way analysis of variance as appropriate.

Effects on Prothrombin Activation by Factor Xa

Plasma defibrinated by Arvin was used as the source of prothrombin. Plasma (0.5 mL) and cephalin (6 μg organic phosphate) were incubated at 37 °C for five minutes. Factor Xa (0.1 to 10 nmol/L final concentration) and calcium chloride were then added. After 15, 30, 45, 60, 90, and 120 seconds of incubation at 37 °C, aliquots of the mixture were removed into EDTA, and the thrombin activity quantitated as above. In addition, the amount of the added factor Xa still measurable at each of these incubation times was also determined. This permitted an estimate to be made of the rate of inactivation of factor Xa in normal plasma. These experiments were also conducted with plasma containing each of the six sulfated polysaccharides.

RESULTS

The effect of each glycosaminoglycan on the TCT and factor Xa activity in vitro and ex vivo are shown in Fig 1. Standard heparin inhibited both factor Xa and thrombin, the octasaccharide only inhibited factor Xa, and dermatan sulfate only inhibited thrombin activity.

Effect of Sulfated Polysaccharides on the Activation of Prothrombin by Factor Xa Added to Normal Plasma

Standard heparin in a dose of 2.5 anti-factor Xa U/kg inhibited thrombus formation by 58%, and in a dose of 10 anti-factor Xa U/kg inhibited thrombus formation by 90% (Fig 2). The octasaccharide in a dose of 10 anti-factor Xa U/kg (which produced comparable anti-factor Xa activities) (Table I) only inhibited thrombus formation by 41%, and doubling the dose to 20 anti-factor Xa U/kg had no further effect. Dermatan sulfate at a dose of 500 μg/kg, inhibited thrombus formation by 95%.

The relationships between the inhibitory effects of each glycosaminoglycan and its associated anticoagulant activity measured ex vivo are summarized in Table 1. The maximum inhibitory effect of standard heparin was achieved with a dose of 10 anti-factor Xa U/kg. This effect was associated with eight- and two-second prolongations in the APTT and TCT, respectively, and with a circulating anti-factor Xa activity of 0.32 U/mL. The dose of the octasaccharide that produced comparable anti-factor Xa activity (0.28 U/mL) had a significantly smaller antithrombotic effect (P < .01). In contrast, dermatan sulfate, at a dose that had no detectable effects on the APTT and TCT, and no anti-factor Xa activity, inhibited thrombus formation by 95%.

Effect of Sulfated Polysaccharides on the Activation of Prothrombin by Factor Xa Added to Normal Plasma

Table 2 shows the relationship between the efficiency of various sulfated polysaccharides to accelerate inactivation of added factor Xa and their ability to inhibit thrombin generation in plasma. For standard heparin, there appeared to be a direct relationship between the potentiation of factor Xa inactivation and...
the inhibition of thrombin generation. The heparin octasaccharide, although very efficient at enhancing the inactivation of factor Xa by plasma, was a very weak inhibitor of thrombin generation. In contrast dermatan sulfate, while a poor accelerator of factor Xa inactivation, was an effective inhibitor of thrombin generation. Similar effects were obtained with the other incubation times.

**DISCUSSION**

Our findings that the octasaccharide was a relative weak antithrombotic agent confirm the observations of Thomas et al.\(^1\) and Ockelford et al.\(^2\). These investigators have observed that heparins with low antithrombin activities are poor antithrombotic agents. A possible explanation for this in vivo effect is provided by the results of our in vitro studies that demonstrate that the heparin octasaccharide was as efficient as standard heparin in accelerating the inactivation of factor Xa but was poor in inhibiting the generation of thrombin activity (Table 2). In contrast, dermatan sulfate, which is known to inhibit thrombin generation but not factor Xa activity in plasma,\(^3,4\) showed marked antithrombotic properties in our experimental model. Dermatan sulfate has been shown to activate heparin cofactor II and not antithrombin III.\(^6,17\) Furthermore, we have shown in plasma that dermatan sulfate has a minimal effect on the inactivation of factor Xa at concentrations (greater than 6.6 \(\mu \text{g/mL}\)) that markedly inhibit thrombin generation (Table 2). Therefore, it appears that the antithrombotic effect of glycosaminoglycans can be achieved by the potentiation of either antithrombin III or heparin cofactor II. The reason for the failure of the antithrombotic dose of dermatan sulfate to prolong the TCT or the APTT is not entirely clear but may reflect the insensitivity of the APTT and TCT to detect both the antithrombotic effect of this glycosaminoglycan and its ability to inhibit thrombin generation in plasma.

**ACKNOWLEDGMENT**

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**REFERENCES**


**Table 1. Minimum Dose SH, OS, and DS Required to Achieve Maximum Antithrombotic Effect In Vivo, and Associated Anticoagulant Activities ex vivo**

<table>
<thead>
<tr>
<th>Heparin Type</th>
<th>Minimum Dose</th>
<th>Maximum Effect (%)</th>
<th>APTT (s)</th>
<th>TCT (s)</th>
<th>Anti-Factor Xa (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH</td>
<td>10 anti-factor Xa U/kg</td>
<td>90 ± 4</td>
<td>8 ± 1</td>
<td>2 ± 1</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>OS</td>
<td>10 anti-factor Xa U/kg</td>
<td>41 ± 13</td>
<td>1 ± 1</td>
<td>0 ± 0</td>
<td>0.28 ± 0.04</td>
</tr>
<tr>
<td>DS</td>
<td>500 µg/kg</td>
<td>95 ± 5</td>
<td>1 ± 1</td>
<td>0 ± 1</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

N ≥ 14; data expressed as mean ± SEM. SH, standard heparin; OS, octasaccharide; DS, dermatan sulfate.

**Table 2. Relationship Between Efficiency of SH, OS, and DS to Accelerate Inactivation of Factor Xa Added to Plasma, and the Inhibition of the Generation of Thrombin Activity in Plasma**

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Standard Heparin Inhibition (%)</th>
<th>Octasaccharide Inhibition (%)</th>
<th>Dermatan Sulfate Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Factor Xa TG</td>
<td>Factor Xa TG</td>
<td>Factor Xa TG</td>
</tr>
<tr>
<td>0.66</td>
<td>20</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>5.6</td>
<td>95</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>40</td>
<td>99</td>
<td>99</td>
<td>10</td>
</tr>
</tbody>
</table>

| Anti-Factor Xa (U/mL) | 0.00 ± 0.00 | 0.28 ± 0.04 | 0.32 ± 0.02 |

The values reported are those obtained one minute after the addition of factor Xa to initiate the generation of thrombin activity in plasma. TG, thrombin generation.
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