Heparan Sulfate and Dermatan Sulfate Inhibit the Generation of Thrombin Activity in Plasma by Complementary Pathways

By Frederick A. Ofosu, Gaman J. Modi, Lindsay M. Smith, Andrew L. Cerskus, Jack Hirsh, and Morris A. Blajchman

Heparan with a low affinity for antithrombin III has previously been demonstrated to inhibit thrombin generation in both normal plasma and plasma depleted of antithrombin III. In addition, standard heparin and heparin with a low affinity for antithrombin III have been demonstrated to have equivalent inhibitory actions on thrombin generation in plasma depleted of antithrombin III. These observations prompted the investigation of the effects of four normal vessel wall glycosaminoglycans (heparan sulfate, dermatan sulfate, chondroitin-4-sulfate, and chondroitin-6-sulfate) on the intrinsic pathway generation of thrombin and factor Xa and on the inactivation of thrombin and factor Xa in plasma. Heparan sulfate inhibited thrombin generation and accelerated the inactivation of added thrombin and factor Xa in normal plasma but not in antithrombin III-depleted plasma. In contrast, dermatan sulfate inhibited thrombin generation in both normal and antithrombin III-depleted plasma. In addition, heparan sulfate was an effective inhibitor of factor Xa generation, while dermatan sulfate was not. Neither chondroitin-4-sulfate nor chondroitin-6-sulfate inhibited the generation of thrombin or factor Xa nor did they accelerate the inactivation of factor Xa or thrombin by plasma. These results suggest that heparan sulfate acts primarily by potentiating antithrombin III, while dermatan sulfate acts by potentiating heparin cofactor II. The inhibition of thrombin generation by heparan sulfate and dermatan sulfate thus appears to occur by complementary pathways, both of which may contribute to the anticoagulation of blood in vivo.

I t has been suggested that the glycosaminoglycans synthesized by endothelial cells and smooth muscle cells contribute to thromboreistance of normal endothelium by inhibiting thrombus formation. Previous studies have indicated some of these vessel wall glycosaminoglycans, principally heparan sulfate, to be capable of potentiating antithrombin III activity. We and others have demonstrated that part of the anticoagulant activity of heparan is mediated independently of antithrombin III. These observations have led us to evaluate the anticoagulant activities of the following glycosaminoglycans, which are known constituents of the vessel wall: heparan sulfate, dermatan sulfate, chondroitin-4-sulfate, and chondroitin-6-sulfate.

MATERIALS AND METHODS

Materials

Rabbit brain cephalin, fatty acid-free bovine serum albumin, porcine skin dermatan sulfate (lot number 109C-2314), chondroitin-4-sulfate (from whale cartilage), chondroitin-6-sulfate (from shark cartilage) and oxyuranus scutellatus venen were obtained from Sigma Chemical Co, St Louis. Porcine mucosal heparins (specific activity 150 USP units/mg) were products of Riker Laboratories, Northridge, Calif and Harris Laboratories, Brantford, Ontario. The chromogenic substrates H-D-Phe-Pip-Arg-pNA (S2238) and CBz-Ile-Glu-Gly-Arg-pNA (S2222), dextran sulfate (average molecular weight 500,000), DEAE-Sephadex A50, and Sepharose 4B were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Human factor IX concentrate was produced by Connaught Laboratories Ltd, Toronto, Ontario. The factor X activator purified from Russell's Viper Venom (RVV-X) was generously provided by Dr Walter Kisiel, University of Washington, Seattle. Arvin was obtained from Berks Pharmaceuticals, Guildford, Surrey, England. Heparan sulfate (HS[AM-2205] National Heart Institute Reference Standard) was a gift from Dr Cifonelli, University of Chicago. Heparan sulfate was an effective inhibitor D-Phe-Pro-Arg CH2CI was obtained from Calbiochem-Behring, La Jolla, Calif.

The Preparation of Thrombin, Factor Xa, and Antithrombin III

Human thrombin, isolated from factor IX concentrate after activation of the prothrombin with Taipan snake venom, had specific activity of 2,500 NIH units/mg protein. Human factor Xa was obtained by the activation of factor X with RVV-X and CaCl2 and separated from the activator by chromatography on DEAE-Sephadex-A50. Factor X was isolated from human plasma by modifications of the methods of di Scipio et al, as we have described previously. The two human proteases were stored in 50% glycerol at -20 °C. Human antithrombin III was isolated from fresh human plasma using the methods of Miller-Andersson et al. The thrombin, factor Xa, and antithrombin III were homogeneous on SDS-polyacrylamide gels.

Effects of Vessel Wall Glycosaminoglycans and Heparin on the Factor Xa-Induced Generation of Thrombin Activity in Plasma

Platelet-poor plasma was prepared from whole blood anticoagulated with 4% sodium citrate, pH 7.0 (nine volumes blood to one volume citrate) by centrifugation at 10,000 g for ten minutes at 4°C. The pooled platelet-poor plasma was derived from 15 to 20 healthy
The activation of prothrombin in normal plasma or plasma depleted of antithrombin III was performed as previously described. Briefly, the plasma was defibrinated by incubation with Arvin (0.15 U/mL of plasma) for ten minutes at 37 °C. The fibrin was removed by centrifugation (5,000 g for two minutes). The defibrinated plasma was used within 30 minutes of preparation.

Thrombin generation in plasma was quantitated in a two-stage assay. In the first stage, 0.275 mL buffer (0.03 mol/L sodium barbiturate, pH 7.4 containing 0.15 mol/L NaCl and 0.1 mg/mL fatty acid-free bovine serum albumin), 0.1 mL CaCl₂ (0.1 mol/L), 0.025 mL cephalin (containing 2 μg organic phosphate), and 0.1 mL of factor Xa (100 ng) were incubated in a 12 × 75-mm plastic test tube at 37 °C for two minutes. Plasma prewarmed to 37 °C (0.5 mL) was then added. In the second stage, 0.2-mL samples of the stage-1 mixture were removed at 15, 30, 45, and 60 seconds and added to 0.8 mL of 10 mmol/L EDTA in 0.03 mol/L sodium barbiturate, pH 7.4, containing 0.15 mol/L NaCl and 0.1 mg/mL fatty acid-free bovine serum albumin preincubated at 4 °C. The amount of thrombin generated at each of the above incubation times was quantitated immediately by adding 0.025 mL of the EDTA-containing sample to 0.975 mL of 0.25 mmol/L solution of S-2238 in 0.03 mol/L sodium barbiturate, pH 7.4, containing 0.15 mol/L NaCl and 0.1 mg/mL fatty acid-free bovine serum albumin. The S-2238 solution had been preincubated at 37 °C in semi-microcuvettes in a Gilford Model 250 Spectrophotometer (Oberlin, Ohio) equipped with a thermal printer attachment. After a one-minute incubation at 37 °C, the rate of amidolysis of the S-2238 by thrombin was quantitated at 405 nm.

The effects of heparan sulfate, dermatan sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, or standard heparin on thrombin generation were determined by adding the glycosaminoglycan to stage 1 of the incubation mixture before the addition of the defibrinated plasma. The glycosaminoglycan concentrations varied from 0.66 to 66 μg/mL.

Effects of Glycosaminoglycans on Factor Xa and Thrombin Generation by the Intrinsic Pathway in Plasma

Activated partial thromboplastin time (APTT) reagent (0.25 mL) was added to 0.5 mL of defibrinated plasma. The mixture was incubated at 37 °C for five minutes, at which time 0.15 mL of 0.03 mol/L sodium barbiturate, pH 7.4, containing 0.15 mol/L NaCl and 0.1 mg/mL fatty acid-free bovine serum albumin and 0.1 mL of 0.1 mol/L CaCl₂ were added simultaneously. Both the buffer and the CaCl₂ solution had been preincubated for five minutes at 37 °C. The amount of thrombin generated in the plasma at 15, 30, 45, 60, 90, and 120 seconds after the addition of CaCl₂ was quantitated as above. Factor Xa generation in an aliquot of the same plasma was also quantitated. For this determination, 0.9 mL of 0.4 mmol/L of S-2222 was the substrate used instead of S-2238, and the amount of the EDTA plasma from stage 1 was 0.1 mL. Factor Xa generation was quantitated over five minutes. The effect of each of the glycosaminoglycans on the intrinsic generation of factor Xa and thrombin was determined by adding the glycosaminoglycan to stage 1 in concentrations of 0.66 to 66 μg/mL. In some experiments, factor Xa generation was quantitated in the presence of 1 μmol/L D-Phe-Pro-Arg CH₂Cl. This chloromethyl ketone has been previously demonstrated to be a specific inhibitor for thrombin. The chloromethyl ketone (1 μmol/L) was added to the EDTA barbiturate buffer before the addition of the plasma subsample.

The Effect of Glycosaminoglycans on the Inactivation of Factor Xa and Thrombin by Plasma

The ability of heparin, heparan sulfate, dermatan sulfate, chondroitin-4-sulfate, or chondroitin-6-sulfate to alter the rate of inactivation of either factor Xa or thrombin by plasma was determined in plasma by comparing the residual factor Xa or thrombin present 15, 30, 45, and 60 seconds after the addition of the purified coagulant protease with that present in defibrinated normal or antithrombin III-depleted plasma in the presence and absence of each of the glycosaminoglycans. The plasma concentrations of the glycosaminoglycans varied from 0.66 to 66 μg/mL. In addition to normal plasma and antithrombin III-depleted plasma, factor V-deficient and prothrombin-deficient plasma were used as the source of the plasma inhibitors of factor Xa and thrombin. Each plasma under study contained 10 mmol/L CaCl₂ in addition to cephalin (2 μg organic phosphate per milliliter of plasma). The generation of thrombin activity in each plasma was also determined as described above after the addition of factor Xa.

Formation of Thrombin-Inhibitor Complexes

Formation of complexes between antithrombin III and thrombin and heparin cofactor II and thrombin in either defibrinated normal plasma or antithrombin III-depleted plasma was determined in polyacrylamide gels using the procedures of Tollefsen et al. The ability of the four glycosaminoglycans to influence the formation of thrombin-plasma inhibitor complexes were determined. Briefly, [125I]-labeled thrombin was diluted with cold thrombin to achieve a specific activity of 20,000 cpm/unit. The [125I]-labeled thrombin (10 μL) was added to 25 μL of defibrinated plasma (diluted one in five in 0.05 mol/L TRIS-HCl, pH 8.0, containing 0.15 mol/L NaCl) to which 15 μL of buffer with or without the glycosaminoglycan had previously been added. After a one-minute incubation at 37 °C, 0.2 mL of the electrophoretic buffer was added. This buffer was 0.05 mol/L TRIS-HCl, pH 8.6 containing 10% glycerol, 1% sodium dodecyl sulfate, 4% mercaptoethanol, and 0.01% bromophenol blue. The sample was then boiled for five minutes. After cooling, 50 μL of the sample was subjected to acrylamide gel electrophoresis.

RESULTS

Effects of the Glycosaminoglycans on Factor Xa-Induced Generation of Thrombin Activity

Dermatan sulfate and heparan sulfate inhibited thrombin generation after the addition of factor Xa to normal plasma (Fig 1). Unlike standard heparin, how-
ever, complete inhibition of thrombin generation was not observed. In antithrombin III-depleted plasma, dermatan sulfate and standard heparin were equally effective inhibitors of thrombin generation (Fig 2). In contrast, no inhibition of thrombin generation could be observed in antithrombin III-depleted plasma containing heparan sulfate (Fig 2). Neither chondroitin-4-sulfate nor chondroitin-6-sulfate inhibited the generation of thrombin when added to either normal or antithrombin III-depleted plasma (data not shown).

Figure 3 shows the effects of heparin, heparan sulfate, and dermatan sulfate on the inactivation of factor Xa added to plasma and the inhibition of thrombin generation in the plasma to which factor Xa had been added. Dermatan sulfate was unable to accelerate the inhibition of the factor Xa added to initiate thrombin generation. Thus, compared with the control plasma, 95% of the added factor Xa was measurable 60 seconds after incubation in normal plasma containing 66 μg dermatan sulfate. However, only 20% of the thrombin that was generated in the control plasma could be detected in the plasma containing 66 μg/mL dermatan sulfate. Heparan sulfate, on the other hand, accelerated the inactivation of factor Xa by normal plasma. The enhanced rate of inactivation of factor Xa in plasma by heparan sulfate was associated with the inhibition of thrombin generation. Similar results were obtained at 15, 30, and 45 seconds. The results obtained with heparin were qualitatively similar to those obtained with heparan sulfate. None of the three glycosaminoglycans was capable of accelerating the inhibition of factor Xa in plasma depleted of antithrombin III.

**Effect on the Inactivation of Factor Xa and Thrombin**

The effects of heparan sulfate, dermatan sulfate, and heparin on the inhibition of thrombin by normal and antithrombin III-depleted plasma are summarized in Table 1. Table 1 also shows the effects of the three glycosaminoglycans on the inactivation of factor Xa by normal and antithrombin III-depleted plasma. All three glycosaminoglycans enhanced the inhibition of thrombin by normal plasma. While both heparin and dermatan sulfate accelerated the inhibition of thrombin in antithrombin III-depleted plasma, heparan sulfate did not. In contrast to the effects on the inactivation of thrombin, dermatan sulfate did not accelerate the ability of normal and antithrombin III-depleted plasma to inactivate factor Xa. In addition, while heparan sulfate and heparin accelerated the rate of inactivation of factor Xa by normal plasma, no acceleration was observed in antithrombin III-depleted plasma. Chondroitin-4-sulfate and chondroitin-6-sulfate did not influence the rate of inactivation of factor Xa or thrombin in either plasma (results not shown).

We considered the possibility that the thrombin formed when factor Xa, CaCl₂, and cephalin were added to plasma could influence the rate of inactivation of factor Xa by competing with factor Xa for antithrombin III. We therefore examined the contribution of thrombin generation to the inactivation of factor Xa by plasma by conducting the experiments in either factor V-deficient or prothrombin-deficient plasma. There were no differences in the rates of inactivation of factor Xa in factor V-deficient or prothrombin-deficient plasma compared with normal plasma (results not shown).

**Inhibition of the Intrinsic Activation of Factor X and Prothrombin by Heparan Sulfate and Dermatan Sulfate**

Figure 4 shows the effects of increasing concentrations of heparan sulfate and dermatan sulfate on the generation by the intrinsic pathway of plasma factor
Xa and thrombin activities in normal plasma. Dermatan sulfate was a relatively poor inhibitor of factor Xa generation. The maximum inhibition obtained with this inhibitor was 25%. In contrast to dermatan sulfate, heparan sulfate was an effective inhibitor of factor Xa generation. Both glycosaminoglycans were effective inhibitors of thrombin generation by the intrinsic pathway. Compared with standard heparin, however, dermatan sulfate and heparan sulfate were poor inhibitors of the APTT of plasma (Table 2).

The contribution of thrombin to the hydrolysis of S-2222 by factor Xa was evaluated by conducting some experiments in the presence of 1 μmol/L D-Phe-Pro-Arg CH₂Cl. No inhibition of the activity of purified factor Xa was observed in the presence of 1 μmol/L D-Phe-Pro-Arg CH₂Cl. In contrast, the amidolytic activity of thrombin on S-2238 was completely inhibited by this concentration of inhibitor. The amidolytic activity of factor Xa generated in plasma by the intrinsic pathway was reduced by only 5% when factor Xa was quantitated in the presence of 1 μmol/L of the chloromethyl ketone inhibitor. There was no difference in the results obtained in experiments on factor Xa generation in the presence of the three glycosaminoglycans whether the chloromethyl ketone was present or not.

### Formation of Thrombin-Inhibitor Complexes

The effects of heparin, dermatan sulfate, and heparan sulfate on the formation of thrombin-plasma inhibitor complexes in defibrinated normal plasma and antithrombin III-depleted plasma were determined after the addition of 125I-labeled thrombin to the various plasmas containing the glycosaminoglycans. The results are shown in Fig 5. In normal plasma containing heparan sulfate, only a thrombin–antithrombin III complex was formed. Heparan sulfate had minimal effect on the formation of thrombin–heparin cofactor II complexes in antithrombin III-depleted plasma. Dermatan sulfate catalyzed only the formation of thrombin–heparin cofactor II complexes in both normal and antithrombin III-depleted plasmas. Standard heparin catalyzed the formation of both thrombin–antithrombin III and thrombin–heparin cofactor II complexes in normal plasma and thrombin–heparin cofactor II complexes in antithrombin III-depleted plasma.

### DISCUSSION

Heparan sulfate and dermatan sulfate are principal glycosaminoglycan constituents of the vessel wall proteoglycan coat. Both glycosaminoglycans have previously been shown to prolong the partial thromboplastin times of normal plasma and to inhibit thrombus.
Fig 5. The effects of standard heparin, dermatan sulfate, and heparan sulfate on the formation of thrombin–antithrombin III (IIa-ATIII) and thrombin–heparin cofactor II (IIa-HCII) complexes in normal and antithrombin III-depleted plasmas. The samples applied to each lane contained approximately 20,000 cpm of 125I-labeled human thrombin (IIe). Lane 1: purified antithrombin III + heparin (0.66 μg/mL); lane 2: normal plasma + 0.66 μg/mL heparin; lane 3: normal plasma + 66 pg/mL heparin; lanes 4 through 8 are normal plasma plus dermatan sulfate (0.66, 6.6, and 66 pg/mL, respectively); lanes 7 through 9 are normal plasma plus heparan sulfate (0.66, 6.6, and 66 pg/mL, respectively); lane 10: antithrombin III-depleted plasma + 66 pg/mL standard heparin; lane 11: antithrombin III-depleted plasma + 66 pg/mL dermatan sulfate; lane 12: antithrombin III-depleted plasma + 66 pg/mL heparan sulfate; lane 13: thrombin only.

Formation in vitro. Heparan sulfate has been shown to have a weak anticoagulant activity when measured by its ability to potentiate the inactivation of thrombin and factor Xa. Dermatan sulfate has been shown to potentiate the inactivation of thrombin by heparin cofactor II. A weak effect on the thrombin–antithrombin III reaction has also been reported; although this may be due to contamination by heparin.

In comparison with heparin, dermatan sulfate and heparan sulfate are relatively poor at prolonging the clotting times of plasma in several clotting tests. Our results indicate that, in spite of their weak effect on the clotting times, dermatan sulfate and heparan sulfate are potent inhibitors of the intrinsic pathway for thrombin generation. The other principle glycosaminoglycans of the vessel wall, chondroitin-4-sulfate and chondroitin-6-sulfate, appear to have no effect on thrombin generation. The cause for the poor correlation between prolongation of clotting times using global clotting tests and inhibition of thrombin generation is not clear. It is possible that global clotting tests are relatively insensitive to the anticoagulant effects of dermatan sulfate and heparan sulfate because the end point (fibrin formation) requires a threshold level of thrombin for detection and is not indicative of the rate of thrombin formation.

Tollefsen et al have recently described in detail the catalytic effect of dermatan sulfate and other glycosaminoglycans on the thrombin–heparin cofactor II reaction in a purified protease-inhibitor system. Our results indicate that, in plasma, the anticoagulant activity of dermatan sulfate likely results from its ability to potentiate the inactivation of thrombin by heparin cofactor II. In contrast, heparan sulfate appears to act primarily by potentiating the activity of antithrombin III, since no inhibition of thrombin generation was demonstrable in antithrombin III-depleted plasma containing heparan sulfate. It is unknown whether dermatan sulfate and heparan sulfate, like heparin, can disrupt the assembly of the prothrombinase and the tenase complexes. Chondroitin-4-sulfate and chondroitin-6-sulfate had no anticoagulant activity in any of the test systems used.

It thus appears that, in plasma, dermatan sulfate and heparan sulfate have complementary modes for inhibiting thrombin generation. Heparan sulfate acts primarily by potentiating the inactivation of thrombin and factor Xa by antithrombin III, while dermatan sulfate appears primarily to potentiate the inhibition of thrombin by heparin cofactor II. The present results, derived from in vitro experiments, may not reflect conditions in vivo. Nonetheless, if the anticoagulant activities of these two vessel wall glycosaminoglycans...
are also a significant feature of the proteoglycans from which they were derived, as the work of Busch and Owen would suggest, they could contribute significantly to the anticoagulation of blood in vivo.

REFERENCES

Heparan sulfate and dermatan sulfate inhibit the generation of thrombin activity in plasma by complementary pathways

FA Ofosu, GJ Modi, LM Smith, AL Cerskus, J Hirsh and MA Blajchman