Heparin, a complex glycosaminoglycan of alternating D-glucosamine and uronic acid residues, is a potent anticoagulant of proven clinical value. Heparin is an effective antithrombotic because its primary target is the serine proteinase, thrombin (IIa), an enzyme central to the maintenance of hemostasis and the processes of thrombosis. Heparin dramatically accelerates the rate of inactivation of IIa by the serpin, antithrombin III (ATIII). It has been proposed that the predominant antithrombotic action of heparin in plasma is via the inhibition of IIa activation of the cofactors factors V and VIII, which in turn inhibits further IIa generation.1-3

Despite its excellent anticoagulant properties, there are some clinical situations in which heparin’s efficacy is limited. Reocclusion of coronary arteries following thrombolytic therapy occurs in one third of patients, despite administration of heparin.4,5 Also, preventing the propagation of venous thrombosis requires higher concentrations of heparin than preventing its formation,6 and 25% of patients still have thrombus extension while receiving heparin treatment.7 Thrombus extension while receiving heparin treatment is thus considered dynamic thrombosis. Our aim was to determine whether fibrin also compromises heparin efficacy in plasma. We found that soluble fibrin ablated the heparin-mediated prolongation of the thrombin time with half-maximal effect at 60 nmol/L fibrin. The fibrin-mediated prolongation of the activated partial thromboplastin time (APTT) was also reduced by fibrin with half-maximal effects at 140 nmol/L fibrin using 0.12 U/mL heparin and 500 nmol/L fibrin using 0.25 U/mL heparin.

Inhibition of Heparin Activity in Plasma by Soluble Fibrin: Evidence for Ternary Thrombin-Fibrin-Heparin Complex Formation

By Kylie A. Hotchkiss, Colin N. Chesterman, and Philip J. Hogg

The ability of heparin to dramatically enhance the inactivation of thrombin (IIa) by antithrombin III (ATIII) in buffer is negated through formation of a IIa-fibrin-heparin ternary complex (Hogg and Jackson, Proc Natl Acad Sci USA 86:2619, 1989; Hogg and Jackson, J Biol Chem 265:241, 1990). IIa, in this ternary complex, is protected from inactivation by ATIII. Our aim was to determine whether fibrin also compromises heparin efficacy in plasma. We found that soluble fibrin ablated the heparin-mediated prolongation of the thrombin time with half-maximal effect at 60 nmol/L fibrin. These findings imply that fibrin is a potent modulator of heparin activity in vivo by inhibiting heparin-catalyzed ATIII complex formation through formation of ternary IIa-fibrin-heparin complexes.

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The mechanism of inhibition of heparin activity by fibrin in plasma was determined by measuring IIa-ATIII complexes by enzyme-linked immunosorbent assay (ELISA). Fibrin was found to inhibit the heparin-catalyzed inactivation of IIa by ATIII with half-maximal effect at 97 ± 19 nmol/L fibrin. Fibrin had no effect on the heparin-catalyzed inactivation of factor Xa by ATIII in plasma, using either standard heparin, a heparinoid preparation (Orgaran; Organon, Lane Cove, Sydney, Australia), or low-molecular weight heparin. These findings imply that fibrin is a potent modulator of heparin activity in vivo by inhibiting heparin-catalyzed ATIII complex formation through formation of ternary IIa-fibrin-heparin complexes.

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which negate the heparin-catalyzed inactivation of IIa by ATIII. Furthermore, the results can explain why standard heparin is not optimal in the treatment of thromboses involving extensive IIa and fibrin formation, and suggest that low-molecular weight heparin will be more effective than standard heparin in preventing and treating such thromboses.

MATERIALS AND METHODS

Chemicals. Gly-Pro-Arg-Pro (GPRP) was synthesized in solid phase on phenylaceticamidomethyl resin using an Applied Biosystem Peptide Synthesizer Model 430A (Applied Biosystem, Sydney, Australia) and t-butyloxycarbonyl chemistry. Hydrogen fluoride cleavage of the fully protected peptide was performed by Auspep Pty, Ltd, Parksville, Australia. Lyophilized crude peptide was solubilized in 0.5 mol/L tris-(hydroxymethyl)-aminomethane hydrochloride, 6 mol/L guanidine hydrochloride, pH 8.0, and purified by C18 reverse-phase chromatography (Delta Pak 15 C18-100A, Millipore Australia Pty, Ltd, Sydney, Australia) using a 0% to 70% linear acetonitrile gradient containing 0.1% trifluoroacetic acid. Peptide purity was greater than 95% based on its chromatographic profile.

Sodium heparin was from David Bull Laboratories Pty, Ltd (Melbourne, Australia) Fragmin from Fisons Pharmaceuticals (Castle Hill, Australia) and Orgaran from Organon Pty, Ltd (Lane Cove, Sydney, Australia). O-Phe-Pro-Arg-chloromethyl ketone (FPR-CH2Cl) was purchased from Calbiochem (San Diego, CA) and hexadimethrine bromide (Polybrene) from Sigma Chemical Co (St. Louis, MO).

Peptidase. Human a-thrombin was supplied by Dr John Fenton (New York State Department of Health, Albany, NY). The active enzyme concentration was determined by active site titration.13 Fibrinogen was prepared from human fresh frozen plasma by a modification of the method of Jacobsen and Kierulf14 as described by Hogg and Jackson.15 Fibrin II monomer (fbnIIIm) was prepared by clotting purified fibrinogen with a-thrombin and solubilizing the fibrin in 0.02 mol/L acetic acid. Plasma is able to support approximately 0.5 μmol/L fbnIIIm in solution. This was tested by adding 20 μL of 5 μmol/L fbnIIIm or 0.02 mol/L acetic acid to 180 μL of plasma, and monitoring turbidity at 405 nm as a function of time using a kinetic microplate reader (Molecular Devices, Menlo Park, CA). No change in turbidity of plasma containing 0.5 μmol/L fbnIIIm relative to control plasma containing 2 nmol/L acetic acid was observed over 10 minutes. Also, the pH of plasma is essentially unchanged by the addition of 2 nmol/L acetic acid in cases where the fibrin concentration exceeded 0.5 μmol/L. fbnIIIm was prevented from polymerizing by 4 mmol/L of the tetrapeptide, GPRP.

Plasma samples. Platelet-poor plasma was prepared from whole blood anticoagulated with 2.2% trisodium citrate followed by centrifugation at 3,000g for 15 minutes.16 Pooled normal plasma was obtained from the Red Cross Blood Bank, Sydney, Australia. Plasma from one patient therapeutically anticoagulated with warfarin was prepared similarly. The plasma had an INR of 5.8 and an activated partial thromboplastin time (APTT) of 40.9 seconds. Plasma from a patient with lupus anticoagulant17 and a prolonged APTT (30.7 seconds) was also used as a control.

Thrombin time (TT) assay. The effect of 34 to 272 nmol/L fbnIIIm on the TT of normal and 0.2 U/mL heparinized plasma was investigated. FbnIIIm and/or heparin or saline as control was added to citrated plasma. Test samples were vortexed and incubated at 37°C for 1 minute. Following incubation, the clotting reaction was initiated by addition of IIa to give a final concentration of 20 nmol/L. The final plasma dilution was 0.53/1. The clotting time was then measured using the ACL 2000 (Coulter Electronics Pty, Ltd, Brookvale, Australia). Addition of 0.02 mol/L acetic acid instead of fbnIIIm to plasma had no effect on the TT in the absence or presence of heparin.

APTT assay. The effect of 34 to 544 nmol/L fbnIIIm on the APTT of 0.12 U/mL and 0.25 U/mL heparinized plasma was investigated. FbnIIIm and/or heparin or saline as control was added to citrated plasma. Fifty microliters of the test plasma sample was incubated with 50 μL Manchester APTT reagent at 37°C for 5 minutes. The APTT determination was initiated with 50 μL of 25 nmol/L CaCl2 and the clotting time measured using the ACL 2000 (Coulter). The final plasma dilution was 0.27/1. Addition of 0.02 mol/L acetic acid instead of fbnIIIm to plasma had no effect on the APTT in the absence or presence of heparin.

Enzyme-linked immunosorbent assay (ELISA) for IIa-ATIII complex. The effect of fbnIIIm on heparin-catalyzed IIa-ATIII complex formation was analyzed by measuring IIa-ATIII complex by ELISA. Test plasma samples were prepared by diluting citrated plasma 0/1 with 0 to 2 μmol/L fbnIIIm/4 nmol/L GPRP and/or 0.05 U/mL heparin or saline as control. Samples were incubated for 1 minute at 37°C. Reactions were initiated by the addition of IIa to a final concentration of 5 nmol/L. Complex formation was allowed to proceed for 15 seconds at 37°C, after which the reaction was terminated by quenching with 477 μmol/L FPR-CH2Cl and 10 mg/mL Polybrene. The IIa-ATIII complex concentration of each test sample was then quantitated via the Behring Enzygnost TAT ELISA assay (Behring, Marburg, Germany). Addition of either 4 nmol/L GPRP or 0.02 mol/L acetic acid instead of fbnIIIm to plasma had no effect on IIa-ATIII complex formation in the absence or presence of heparin.

Chromogenic assay for factor Xa activity. The effect of 2 μmol/L fbnIIIm on the heparin-catalyzed inactivation of factor Xa by ATIII in plasma was compared using three types of heparin, standard unfractionated heparin, low-molecular weight heparin (Fragmin), and a heparinoid (Orgaran). Test plasma samples were prepared by diluting pooled normal plasma 0/1 with 2 μmol/L fbnIIIm/4 nmol/L GPRP and/or 0.8 U/mL heparin, or saline as control. Factor Xa activity was subsequently analyzed for each test sample using the Stachrom Factor Xa kit (Diagnosticus Stago, Asnières-sur-Seine, France). The test principle is based on factor Xa inhibition by heparin-ATIII complexes. A known excess of purified bovine factor Xa is added to the plasma sample. After incubation for 30 seconds, the remaining factor Xa in the sample is measured using a specific chromogenic substrate. For clarity of presentation, the results are presented in terms of percentage of the factor Xa activity in control plasma. Addition of either 4 nmol/L GPRP or 0.02 mol/L acetic acid instead of fbnIIIm to plasma had no effect on factor Xa activity in the absence or presence of heparin.

RESULTS

Effect of fbnIIIm on the heparin-mediated prolongation of the TT and APTT. The effect of fbnIIIm on the heparin-mediated prolongation of the TT of normal plasma is described in Fig 1. Normal plasma clotted with 20 nmol/L IIa was characterized by a TT of 10 ± 0.4 seconds. Heparin at 0.2 U/mL markedly prolonged the TT of normal plasma to 240 ± 6.3 seconds. The heparin-mediated prolongation of the TT was ablated by 272 nmol/L fbnIIIm, restoring the TT to control times. The effect of fbnIIIm was potent with half-maximal inhibition achieved at 60 nmol/L fbnIIIm. FbnIIIm alone, 272 nmol/L, did not depress the TT of normal plasma, 10 ± 0.1 seconds, implying that the attenuation of the prolonged TT is due to interference of heparin activity by fbnIIIm. The effects of fbnIIIm on heparin activity were inde-
Effect of fbnIIIm on the heparin-mediated prolongation of the TT. The TT (seconds) was measured as described in the Methods. The TT in the absence of heparin was $10 \pm 0.4$ seconds (---), which was unaffected by the presence of 272 nmol/L fbnIIIm, $10 \pm 0.1$ seconds (data not shown). Half-maximal inhibition occurs at 60 nmol/L fbnIIIm. The error bars represent the mean ± SD of triplicate experiments.

Fig 1. Effect of fbnIIIm on the heparin-mediated prolongation of the TT. The TT (seconds) was measured as described in the Methods. Control plasma had an APTT of 20 ± 0.7 seconds (---), which was unaffected by the presence of 540 nmol/L fbnIIIm, 19 ± 0.6 seconds (data not shown). Reactions contained either 0.12 U/mL or 0.25 U/mL heparin (○) or 272 nmol/L heparin (□). FbnIIIm attenuated the heparin-mediated prolongation of the APTT with half-maximal inhibition at approximately 140 nmol/L and 500 nmol/L fbnIIIm, respectively. The error bars represent the mean ± SD of triplicate determinations.

Effect of fbnIIIm on the heparin-catalyzed inactivation of IIa by ATIII in plasma. The effect of fbnIIIm on heparin-catalyzed IIa-ATIII complex formation in normal plasma is described in Fig 3. A concentration of IIa-ATIII complex of $0.7 \pm 0.1$ nmol/L resulted from the addition of 5 nmol/L IIa to normal plasma, which was unaffected by the presence of 2 µmol/L fbnIIIm, $0.5 \pm 0.1$ nmol/L (data not shown). Heparin at 0.05 U/mL elevated the plasma concentration of IIa-ATIII complex to $3.3 \pm 0.3$ nmol/L. The heparin-mediated enhancement of IIa-ATIII complex formation was ablated

Fig 2. Effect of fbnIIIm on the heparin-catalyzed inactivation of IIa by ATIII. IIa-ATIII complex concentration was determined by ELSA as described in the Methods. Reactions contained 0.05 U/mL heparin and were initiated by the addition of IIa to a final concentration of 5 nmol/L. A concentration of IIa-ATIII complex of $0.7 \pm 0.1$ nmol/L resulted from the addition of 5 nmol/L IIa to normal plasma (---), which was unaffected by the presence of 2 µmol/L fbnIIIm, $0.5 \pm 0.1$ nmol/L (data not shown). Fit of the data to Equation 1 by least-squares regression with $K_d$ the unknown parameter results in a $K_d$ of 97 ± 19 nmol/L. The error bars represent the mean ± SD of quadruplicate experiments.

Fig 3. Effect of fbnIIIm on the heparin-catalyzed inactivation of IIa by ATIII. IIa-ATIII complex concentration was determined by ELSA as described in the Methods. Reactions contained 0.05 U/mL heparin and were initiated by the addition of IIa to a final concentration of 5 nmol/L. A concentration of IIa-ATIII complex of $0.7 \pm 0.1$ nmol/L resulted from the addition of 5 nmol/L IIa to normal plasma (---), which was unaffected by the presence of 2 µmol/L fbnIIIm, $0.5 \pm 0.1$ nmol/L (data not shown). Fit of the data to Equation 1 by least-squares regression with $K_d$ the unknown parameter results in a $K_d$ of 97 ± 19 nmol/L. The error bars represent the mean ± SD of quadruplicate experiments.
by fbnIIm. To calculate the half-maximal effect of fbnIIm, the data of Figure 3 were fit to Equation 1:

\[
[Ia-ATIII] = \left[ F \right] \cdot \left( \frac{[Ia-ATIII]_{\text{min}}}{[Ia-ATIII]_{\text{max}}} \right) \left( \frac{K_d + [F]}{} \right) + [Ia-ATIII]_{\text{max}} \quad (1)
\]

where \([F]\) is the free concentration of fbnIIm, \(K_d\) is the concentration of fbnIIm at half-maximal inhibition, \([Ia-ATIII]_{\text{max}}\) is the concentration of Ia-ATIII complex in the absence of heparin and presence of 2 \(\mu\)mol/L fbnIIm, 0.5 \(\mu\)mol/L, and \([Ia-ATIII]_{\text{max}}\) is the concentration of Ia-ATIII complex in the presence of 0.05 U/mL heparin, 3.3 \(\mu\)mol/L. Fit of the data of Fig 3 to Equation 1 by least squares regression \(^{18}\) with \(K_d\) the unknown parameter results in a \(K_d\) of 97 \(\pm\) 19 \(\mu\)mol/L. This value is similar to the potency of fbnIIm on the heparin-mediated prolongation of the TT, 60 nmol/L (Fig 1).

Effect of fbnIIm on standard heparin, low-molecular weight heparin, and heparinoid-mediated factor Xa inactivation by ATIII in plasma. Comparison of the relative potencies of standard heparin, low-molecular weight heparin (Fragmin), and a heparinoid preparation (Orgaran) in catalyzing plasma factor Xa inactivation by ATIII and the susceptibility of their activity to modulation by fbnIIm is shown in Fig 4. The degree of heparin-mediated factor Xa inactivation is expressed in terms of the percentage of remaining factor Xa activity. In the absence of heparin, plasma had maximal factor Xa activity, which was not significantly altered by the presence of 2 \(\mu\)mol/L fbnIIm. Standard heparin, Orgaran and Fragmin at 0.8 U/mL depressed plasma factor Xa activity. Furthermore, the reduction in factor Xa activity was maintained in the presence of 2 \(\mu\)mol/L fbnIIm, indicating that the heparin-catalyzed inactivation of factor Xa by ATIII is not susceptible to modulation by fbnIIm.

DISCUSSION

The results demonstrate that soluble fbnIIm is a potent inhibitor of heparin activity in plasma as indicated by the TT and APTT assays. The heparin-mediated prolongation of the TT and APTT is ablated by fibrin with half-maximal effects at 60 nmol/L and 140 to 500 nmol/L fbnIIm, respectively. The less potent effect of fbnIIm on the heparin-mediated prolongation of the APTT is probably due to some protection of Ila from binding fbnIIm and heparin by the prothrombinase complex or to the lack of effect on factor Xa (see below), therefore leading to substantially more Ila than would be used in the TT. To determine the mechanism of this effect of fbnIIm on heparin activity in plasma, Ila-ATIII complex formation was measured as a function of heparin and fbnIIm concentrations. The heparin-catalyzed elevation of plasma Ila-ATIII complex concentration was attenuated by fbnIIm to control levels measured in the absence of heparin. Half-maximal inhibition was achieved at 97 \(\pm\) 19 nmol/L fbnIIm, which correlates with the effect of fbnIIm in the TT assays. This value is similar to the half-maximal effect of fbnIIm on heparin-catalyzed inactivation of Ila by ATIII measured with purified proteins in a buffered system, 20 nmol/L.\(^9\)

The effects of fbnIIm on heparin activity in plasma reported herein parallel those of fbnIIm on heparin activity in a buffered system.\(^9\) In both the buffered and plasma systems fbnIIm essentially negates the anti-IIa effects of heparin and the half-maximal values for fbnIIm are similar, 20 nmol/L in buffer\(^7\) and 60 to 97 nmol/L in plasma. The potency of the effect of fbnIIm argues against the trivial explanation that fbnIIm is simply competing with ATIII for binding heparin. An example of a competitive heparin binding protein that competes effectively with ATIII and, therefore, makes heparin unavailable as a catalyst for inactivation of Ila by ATIII, is platelet factor 4. However, unlike platelet factor 4, fbnIIm binds heparin weakly with a dissociation constant of 5.7 \(\mu\)mol/L.\(^9\) Based on this dissociation constant, 300 nmol/L fbnIIm will have bound only 5% of the heparin in the TT and Ila-ATIII ELISA assays, whereas 300 nmol/L fbnIIm essentially ablated heparin activity in both these systems (equation 3 of reference 12 was used for this calculation).

These results are in accordance with the findings of Hogg and Jackson\(^1,12\) on the effects of fbnIIm on heparin activity in a buffered system. Therefore, the ternary complex model they proposed as the mechanism for the effect of fibrin most likely also applies in plasma. This ternary complex model is illustrated in Fig 5. In this model, fibrin exerts its effect on heparin activity by interfering with productive ternary Ila-heparin-ATIII complex formation, a prerequisite for efficient heparin action. This model also indicates that Ila and heparin both interact with fibrin through the central E domain. Support for this proposal comes from the following considera-
tively stained samples, fibrinogen is a trinodular structure containing two terminal D domains and one central E domain, otherwise referred to as the amino terminal disulfide knot. Studies of the binding of IIa to proteolytic fragments and mutated recombinant and naturally occurring mutants of fibrinogen indicate that IIa binds to the central E domain.

The concentration of soluble fibrin required to neutralize heparin activity in plasma is well within the expected range for the microenvironment of a thrombus. It is likely that the concentration of fibrin at a site of injury, both soluble and polymerized, will at least approach the plasma fibrinogen concentration, 6 μmol/L. Therefore, half-maximal inhibition of heparin activity is expected to occur when only 1% of the local fibrinogen concentration has been converted to fibrin.

IIa also interacts with fibrin polymer and heparin to form a IIa-fibrin polymer-heparin complex and Weitz et al have shown that IIa bound to a plasma derived fibrin clot is refractory to inactivation by heparin-ATIII. Therefore, it is likely that fibrin polymer, like fibrin monomer, also inhibits heparin activity in plasma through formation of a ternary complex with IIa and heparin. Okwusidi et al have shown that the rate of heparin-catalyzed IIa-ATIII complex formation in plasma was enhanced up to twofold when plasma was defibrinated with Arvin. These investigators attributed this effect of Arvin to inhibition of heparin activity by soluble fibrin. In our opinion, the following explanation of their results is more likely. The effects they observed probably resulted from depletion of plasma fibrinogen by Arvin, thereby making it unavailable as a substrate for IIa. In other words, less fibrinogen was available to compete with ATIII for binding to the active site of IIa and the rate of IIa-ATIII complex formation increased. This hypothesis predicts that the rate of IIa-ATIII complex formation would also be faster in Arvin-treated plasma in the absence of heparin, and this is what Okwusidi et al found. Also, it is not unlikely that Arvin-derived fibrin may, in fact, be comparable to thrombin-derived fibrin in complexing with IIa and heparin and be active in inhibiting heparin activity.

IIa binds to approximately 300,000 glycosaminoglycan binding sites per endothelial cell with a dissociation constant in the low nanomolar range. This observation prompted Hogg and Jackson to suggest that endothelial cell surface glycosaminoglycans, like heparin, might also bind IIa and fibrin in a ternary complex and they proposed that formation of these complexes might be a mechanism whereby IIa is restricted to the microenvironment of the injury site. The evidence from these studies that IIa binds fibrin and heparin in plasma supports this hypothesis.

Reocclusion of coronary arteries following thrombolytic therapy and, in particular, the propagation of deep vein thromboses are dynamic thrombotic events usually characterized by extensive IIa and fibrin formation. Therefore, based on our results, it is not surprising that standard heparin has limited antithrombotic efficacy in these clinical situations. However, fibrin has no effect on the heparin-catalyzed
inactivation of factor Xa by ATIII using either standard heparin, a heparinoid preparation or low-molecular weight heparin. Because low-molecular weight heparin has greater anti-Xa activity than anti-IIa activity, and because factor Xa inactivation is not inhibited by fibrin, these results suggest that low-molecular weight heparin may be more effective than standard heparin in preventing and treating the abovementioned thromboses. Indeed, recent clinical data comparing the efficacy of standard versus low-molecular weight heparin in the treatment and prevention of deep vein thromboses suggests that low-molecular weight heparin is a more effective anticoagulant for the treatment of this condition.\textsuperscript{22-24}

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INHIBITION OF HEPARIN ACTIVITY BY FIBRIN

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Inhibition of heparin activity in plasma by soluble fibrin: evidence for ternary thrombin-fibrin-heparin complex formation

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