Fibrin-Targeted Recombinant Hirudin Inhibits Fibrin Deposition on Experimental Clots More Efficiently Than Recombinant Hirudin

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Background Although the indirect thrombin inhibitor heparin and the more potent direct inhibitor hirudin are useful in preventing thrombosis, a substantial opportunity remains for improving the thrombus selectivity of thrombin inhibitors.

Methods and Results To explore the effect of targeting an antithrombin to the surface of a clot, we covalently linked recombinant hirudin to the Fab' (or IgG) of a monoclonal antibody (59D8) that selectively binds to an epitope on fibrin that becomes exposed only after thrombin cleaves fibrinopeptide B. Antibody-coupled hirudin bound to an immobilized peptide of the fibrin β-chain amino-terminal sequence and inhibited the peptidolytic activity of thrombin more efficiently than free hirudin. Thrombin inhibition dependent on binding to immobilized fibrin monomer was enhanced 1100-fold (P<.0001). Hirudin–59D8 Fab' was 10 times more effective than hirudin in inhibiting fibrin deposition on experimental clot surfaces in fibrinogen solution (P<.0001) and human plasma (P<.0001). The more effective inhibition of thrombin by the conjugate was supported by significantly diminished concentrations of fibrinopeptide A in the plasma supernatant of the clot (P=0.0001). Inhibition of clotting by an uncoupled mixture of hirudin and 59D8 Fab' was indistinguishable from that by hirudin alone, indicating that the conjugate’s greater inhibitory activity was due to the covalent linkage between antibody and hirudin.

Conclusions Fibrin-targeted hirudin (in comparison with unmodified hirudin) significantly reduces fibrin deposition on the surface of experimental clots. (Circulation. 1994;90: 1956-1963.)

Key Words • pharmacology • anticoagulants • thrombosis

Using plasminogen activators to recanalize occluded coronary arteries significantly reduces mortality in patients suffering from acute myocardial infarction. Nonetheless, thrombolytic therapy is still limited by the relative resistance of arterial clots to lysis and a high rate of thrombotic reclosure. Acute thrombotic reclosure is also an important setback after otherwise successful percutaneous transluminal coronary angioplasty, and it constitutes a formidable problem after the placement of intracoronary stents. Adjunctive therapy with the currently available antplatelet and anticoagulant agents has shown only modest benefit in preventing rethrombosis after thrombolytic therapy, and there is as yet no clinically validated therapeutic approach to restenosis after angioplasty.

Thrombin plays a central role in the development of arterial clots as the generator of fibrin and, perhaps more importantly, as the most potent activator of platelets. Heparin, which inhibits thrombin indirectly by activating antithrombin III, has been shown in experimental studies to have only a limited antagonistic effect on the action of thrombin. Recently, a number of direct inhibitors of thrombin, such as hirudin, have been shown to be more effective than heparin in preventing platelet-dependent arterial thrombosis and rethrombosis after thrombolytic reperfusion in animals. The underlying mechanisms for their greater efficacy include the susceptibility of clot-bound thrombin to inactivation by inhibitors independent of antithrombin III and the resistance of these antithrombin III–independent inhibitors to endogenous inactivators.

We previously reported that plasminogen activators targeted to the site of a thrombus by conjugation to an antifibrin or antiplatelet antibody have greatly enhanced thrombolytic potency and specificity in vitro and in vivo. Because of the thrombus selectivity of these antibody-targeted plasminogen activators and the diminished fibrinogen consumption they induce, split products that result from the proteolysis of fibrinogen by nonselective plasminogen activators (such as streptokinase) would probably be produced in smaller amounts. These fibrinogen split products, which have an anticoagulant effect, are believed to confer some protection against rethrombosis after streptokinase therapy. A thrombus-targeted anticoagulant would be a useful adjunct for preventing rerecumbrosis should a highly selective plasminogen activator be used in thrombolytic therapy. Also, a targeted anticoagulant would offer the potential of effecting thrombin inhibition in the vicinity of a thrombus without altering systemic homeostatic parameters.

In the present study, we evaluate the relative effects of hirudin and conjugates composed of hirudin and an...
antifibrin antibody or hirudin and the Fab' of that antibody. The antibody, 59D8, binds selectively to the new amino terminus of the fibrin β-chain that becomes exposed after thrombin cleaves fibrinopeptide B.24 Because exposure of this epitope is an early event in the conversion of fibrinogen to fibrin, it is likely that the fibrin-targeted antithrombin conjugate would accumulate at the very site of thrombin action.

Methods

Materials

Recombinant hirudin (r-hirudin LU 52369, with a specific activity of 17 000 antithrombin units (ATU)/mg) was a gift from Knoll AG. The preparation and purification of monoclonal antifibrin antibody 59D8 have been described.16,21

The synthetic peptides Bβ(15-21) (β-peptide) and Bβ(15-21)-Cys were the gifts of Dr Gary Matsueda of the Bristol-Myers Squibb Pharmaceutical Research Institute. H-D-Phenylalanyl-l-pipecolyl-l-arginine-p-nitroanilide dihydrochloride (chromogenic substrate S-2238) was obtained from Chromogenix, N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) from Pierce Chemical, and [3H]-labeled fibrinogen (IBRIN) from Amersham. Fresh-frozen plasma was purchased from the University of Heidelberg blood bank and the German Red Cross. All other chemicals came from Sigma. The ELISA-fibrinopeptide A (FPA) assay was purchased from Boehringer Mannheim.

Preparation of Hirudin–59D8 IgG Conjugate

Disulphide-linked hirudin–IgG conjugate was prepared by reacting an SPDP derivative of hirudin with 2-iminothiolane–substituted antibody 59D8. Hirudin (8 mg, supplied freeze-dried) was dissolved in 2 mL of 50 mmol/L sodium carbonate buffer (pH 8.5), and 2 mL of n-propanol was added to the solution. SPDP in a threefold molar excess (at 20 mmol/L in absolute ethanol) was added dropwise to the solution, and the mixture was stirred gently at room temperature for 30 minutes, after which it was applied to a Sephadex G-25 column (1.6×90 cm) equilibrated with PBS (0.1 mol/L NaH₂PO₄ and 0.1 mol/L NaCl, pH 7.4). Peak protein fractions were pooled and analyzed for 2-pyridyldisulphide content,22 which typically showed 0.6 to 1.5 residues per hirudin molecule. The substitution level was intentionally kept low to limit the loss of hirudin activity and to avoid the formation of higher-molecular-weight aggregates. The specific activity of SPDP-modified hirudin was on average 53% that of unmodified hirudin.

Antifibrin antibody 59D8 (4 mg/mL in 0.14 mol/L NaCl, 3.7 mmol/L NaH₂PO₄, and 1 mmol/L KCl, pH 7.4) was mixed in equal amounts (vol/vol) with a 1000-fold molar excess of 2-iminothiolane in 25 mmol/L sodium borate (pH 9.3). The reaction was allowed to continue for 25 minutes at room temperature with gentle stirring. Excess iminothiolane was removed by gel filtration on Sephadex G-25 preequilibrated with PBS (pH 6.6). A 10-fold molar excess of the SPDP-modified hirudin was then mixed with the iminothiolated antibody and stirred gently at room temperature for 5 hours. At this point, the reaction was stopped by the addition of a 10-fold molar excess (relative to the hirudin concentration) of iodoacetamide in 0.1 mol/L NaH₂PO₄ (pH 8.0).

Preparation of Hirudin–59D8 Fab' Conjugate

The (Fab')₂ of antibody 59D8 was obtained by papain cleavage.17 It was then affinity-purified on a Gly-His-Arg-Pro-Leu-Asp-Lys-(Cy5)-maleimidobenzoylsuccinimide ester (MBS)-lysine-Sepharose column (β-peptide–Sepharose).17 The eluate of the column (0.2 mol/L glycine, pH 2.8) was then dialyzed against PBS. The eluate contained only (Fab')₂, and traces of Fab' or Fab (as assessed by SDS-PAGE). 59D8 (Fab')₂ was reduced at room temperature for 18 hours in 1 mmol/L 2-mercaptoethanol, 1 mmol/L EDTA, and 10 mmol/L sodium arsenate, followed by the addition of solid Ellman’s reagent to a concentration of 5 mmol/L. After 3 hours at room temperature, excess reagent was removed from the mixture by gel filtration on a Sephadex G-25 column (30×2 cm) equilibrated with 0.1 mol/L sodium phosphate (pH 6.8). In this protected form, 59D8 Fab' has remained structurally and functionally intact under sterile conditions at 4°C for at least 1 year.

The thiol form of 59D8 Fab' was regenerated by treatment with 10 mmol/L 2-mercaptoethanol for 30 minutes at room temperature, followed by gel filtration. The thiol form (at 1 mg/mL) was then mixed with SPDP-modified hirudin (1 mg/mL) and allowed to react for 16 hours at room temperature. To minimize the amount of uncoupled 59D8 Fab' in the final preparation, we maintained a 10-fold molar excess of hirudin in the mixture. The reaction was stopped by the addition of an excess of iodoacetamide, as described above.

Purification of Hirudin–59D8 Conjugates

Hirudin–59D8 IgG conjugate and hirudin–59D8 Fab' conjugate were both partially purified from their reaction mixtures by affinity chromatography on a β-peptide–Sepharose column (lysine-Sepharose to which the β-peptide, against which antibody 59D8 had been raised, was coupled through a cysteine residue with MBS). The β-peptide–Sepharose column retained conjugate and unconjugated 59D8 IgG (or 59D8 Fab') but not uncoupled hirudin. The eluate (0.2 mol/L glycine, pH 2.8) from this column was dialyzed into PBS and stored under sterile conditions at 4°C. Alternatively, uncoupled hirudin was separated by gel filtration on Sephadex G-100.

Electrophoresis and Densitometric Scanning

SDS-PAGE was performed according to the method of Laemmli.23 and proteins were visualized by staining with Coomassie brilliant blue G-250.24 The gels were scanned at 633 nm with an LKB Ultrascan XL laser densitometer. Because the correlation between Coomassie brilliant blue G-250 binding and protein content was linear in our hands only between 1 and 10 µg, we determined the ratio of 59D8 IgG to hirudin–59D8 Fab' by measuring optical density at 405 nm. For these calculations we assumed that Coomassie brilliant blue G-250 staining was equal for all proteins examined. Measurements were obtained from redissolved gels that resolved hirudin-conjugated 59D8 IgG and unconjugated 59D8 IgG or hirudin-conjugated 59D8 Fab’ and unconjugated Fab'.

Protein Concentrations

Protein concentrations were determined according to Lowry et al25 (BSA was used as standard) or by measuring optical density at 280 nm.

Measurement of Thrombin Inhibition by Hirudin and Hirudin–Antibody Conjugates (S-2238 Assay)

The proteolytic activity of thrombin can be measured by cleavage of the peptide substrate S-2238, which results in a colored product that can be quantified by reading absorbance at 405 nm. The inhibitory effect (on the activity of thrombin) of hirudin or hirudin–antibody conjugate can then be determined as a function of the degree of inhibition of S-2238 cleavage.

In the S-2238 assay, 20 µL of thrombin solution (2.5 U/mL water) was added to 100 µL of sample (hirudin or hirudin–59D8 conjugate) in assay buffer (20 mmol/L sodium dihydrogen carbonate, 0.15 mol/L NaCl, and 0.1% BSA, pH 7.4) and incubated at room temperature for 10 minutes. Then 50 µL of chromogenic substrate S-2238 (0.833 mg/mL) was added. After exactly 5 minutes of incubation, the reaction was stopped by the addition of 50 µL of 20% acetic acid. Absorbance was measured at 405 nm and compared with that of a thrombin
standard. A decrease in absorbance was linearly related to hirudin concentrations in the range of 0 to 0.6 U/mL. Samples showing higher activity were diluted so that they could be measured within this range.

**β-Peptide Assay**

This assay was used to compare the activities of hirudin and hirudin–antibody conjugate bound to microtiter plates that had been coated with β-peptide. The assay also measures inhibition of S-2238 cleavage by thrombin, although in this instance the inhibitor is bound to the plastic of the microtiter plate instead of being in solution.

One hundred microliters of coating buffer (20 mmol/L Tris, 50 mmol/L NaCl, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, pH 7.4) containing 0.005 mg of β-peptide was applied to each well of a 96-well microtiter plate. After the coating step, the plate was washed five times with coating buffer supplemented with 0.05% Tween 20 and then three times with water. Free binding sites were blocked overnight at 4°C by the addition of 250 µL of BSA (2% wt/vol in coating buffer) per well. The washing step was repeated, and aliquots of hirudin or hirudin–59D8 conjugate (IgG or Fab, measured by S-2238 assay) in 0.1 mol/L NaCl and 0.1 mol/L NaHPO₄ (pH 7.4) were applied in 100 µL of buffer per well and incubated for 2 hours at room temperature.

After the microtiter plate had been washed seven times with modified coating buffer (20 mmol/L Tris, 0.5 mol/L NaCl, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, and 0.2% Tween 20, pH 7.4) and three times with water, 100 µL of assay buffer (20 mmol/L NaHPO₄, 0.15 mol/L NaCl, and 0.1% BSA, pH 7.4) were applied to each well, and 20 µL of thrombin solution (1.25 U/mL) was allowed to incubate for 1 hour at room temperature. Then 50 µL of chromogenic substrate S-2238 (0.83 mg/mL) was applied to the plate, and the reaction was stopped 20 minutes later by the addition of 50 µL of 20% acetic acid. Absorbance in each well was read at 405 nm. A decrease in absorbance reflected an increase in hirudin activity.

**Fibrin-Sepharose Assay**

The purpose of this assay was to compare the activities of hirudin and hirudin–antibody conjugate bound to microtiter plates with some modification. Fresh-frozen plasma from five donors was pooled, divided into aliquots, and refrozen. Before each experiment, plasma was ultracentrifuged at 30 000 rpm for 60 minutes in a Beckman SW 40 rotor to obtain platelet-free plasma (<1000 platelets/µL). Immediately before each experiment, inhibition of thrombin peptidase activity by hirudin and the hirudin conjugate was determined, and appropriate dilutions were made so that inhibition of thrombin peptidase activity was identical for each experimental point. Platelet-free plasma clots were made by adding to plasma, in the following sequence, 125I-labeled human fibrinogen (20 000 cpm/mL plasma), 0.5 mol/L CaCl₂ (0.05 mol/L final concentration), and thrombin (0.5 NIH units/mL plasma). Immediately after the addition of thrombin, the solution was drawn into Silastic tubing (4-mm ID) and allowed to clot for 2 hours at 37°C. The tubing was then cut into 1.8-cm sections to yield clots of about 0.2 mL. The clots were removed from the tubing, and each was placed in a plastic vial and washed three times with 2 mL of 0.15 mol/L NaCl. 125I-radioactivity was measured in a gamma counter, and only clots within 5% of the mean count value were used in the experiments.

The clots were then resuspended in 100 µL of TBS (0.1 mol/L Tris and 0.1 mol/L NaCl, pH 7.4). Experiments were initiated by the addition of 100 µL hirudin (or conjugate) solution for which inhibition of thrombin peptidase activity had been determined. Then 200 µL fibrinogen solution (8 mg/mL) trace-labeled with 125I-fibrinogen (250 000 cpm/mL) was added. The final fibrinogen concentration in the assay was 4 mg/mL (within the physiological range). At predetermined intervals, clots were removed from the test tubes and washed three times with 2 mL of 0.15 mol/L NaCl, and radioactivity was measured in a gamma counter. Clots incubated without thrombin inhibitor (the NaCl controls) often adhered tightly to the wall of the test tube and could not be removed completely.

**Inhibition of Thrombus Growth in Human Plasma In Vitro**

In this assay, which was carried out essentially as described in the preceding section, clots were incubated with 200 µL of platelet-free plasma (instead of 200 µL of fibrinogen solution) that had been trace-labeled with 125I-fibrinogen (250 000 cpm/mL).

**Measurement of Fibrinopeptide A in Human Plasma**

In some experiments, the inhibition of thrombus growth in human plasma was followed as described above, and the concentration of fibrinopeptide A was measured in the plasma supernatant. At various times, thrombin activity was stopped by the addition of hirudin (100 ATU in 100 µL) to the plasma (to a total volume of 500 µL). This plasma solution (100 µL) was then used, after an appropriate dilution with 0.9% NaCl, to determine the amount of fibrinopeptide A by ELISA (ELISA-FPA, Boehringer Mannheim). We followed the manufacturer’s instructions exactly.

**Statistical Analysis**

The dose-response curves in Figs 1 and 2 were compared pairwise (hirudin–59D8 conjugate versus unconjugated hirudin control within each assay) by fitting each curve with the antilogit function

\[
% \text{thrombin inhibition (ATU)} = \frac{B_0 e^{\left[\ln(\text{ATU}) - \ln(\text{ATU}_0)\right]}}{1 + e^{B_0 \left[\ln(\text{ATU}) - \ln(\text{ATU}_0)\right]}}
\]

where \(B_0\) is the ultimate thrombin inhibition at infinite concentration of inhibitor, \(B_1\) is the rate constant for the increase in inhibition with increasing concentration (ATU) of inhibitor, and \(\text{ATU}_0\) is the concentration of inhibitor (in ATU/mL) at which one half the ultimate (\(B_0\)) inhibition is achieved. For each curve, the three parameters \(B_0\), \(B_1\), and \(\text{ATU}_0\) and the variances for each parameter were estimated by use of the FIT FUNCTION procedure of the RS/1 Data Analysis Software.
Fig 1. Graphs showing thrombin inhibition as a function of binding to immobilized target molecule (β-peptide). Hirudin-dependent inhibition of thrombin-mediated cleavage of chromogenic substrate S-2238 was recorded at 405 nm and is expressed as a percentage of inhibition of total thrombin added. A, Hirudin-59D8 IgG conjugate (●) versus hirudin (○). B, Hirudin-59D8 Fab' conjugate (●) versus hirudin (○). Each point represents the mean of three independent experiments.

(BBN Software Products). Corresponding parameters (B₁, B₂, ATUₙ) for each curve were compared by Student's t test.

The time-course curves describing fibrin deposition in Figs 3 through 6B and Fig 6A and fibrinopeptide A generation in Fig 6B were compared by batching y-axis values for each curve across all time points and then, after confirming by one-way ANOVA (ANOVA ONEWAY procedure, RS/1 software) that significant differences existed among some of the curves, performing t tests on the mean fibrin deposition values (in micrograms) or the mean fibrinopeptide A generation values (in micrograms per clot) for each curve. This procedure served as a means of protecting against detecting excessive differences arising from multiple comparisons. Because significant nonlinearities were found in the time-course curves and only three time points were sampled, it was not possible to analyze covariance.

The dose-response curves in Figs 3, 4, and 5 were compared by first performing linear regression analysis of fibrin deposition versus dose of inhibitor for each inhibitor separately and then comparing the two estimated parameters of the regression lines by the t test. The FIT FUNCTION procedure (RS/1 software) was used to estimate the variances of the linear regression parameters.

The time-course curves in Fig 7 were sufficiently linear to permit ANCOVA, with time as a covariate. This analysis tested the hypothesis that the time course of fibrin deposition was identical among the various inhibitors (with and without the NaCl control). The ANCOVA was followed by pairwise planned comparisons (linear contrasts) of the various inhibitor curves (P4V procedure, BMPD Statistical Software, University of California Press, Berkeley and Los Angeles, 1990).

All error estimates cited in the "Results" are standard deviations of the estimates. Potency differences are expressed by the ratios of the estimated ATUₙ parameters.

Results
Characterization of Hirudin-59D8 IgG and Hirudin-59D8 Fab' Conjugates

Hirudin is sensitive to SPDP modification because an intact lysine-47 is essential for its effect as an antithrombin. Since the pK of the ε-amino group is higher than that of the α-amino group at the amino terminus, a reaction pH higher than the pK of the ε-amino group would favor the reaction of SPDP with the amino terminus (Dr Schweden, BASF, personal communication). Therefore, we intentionally kept the concentration of SPDP low so as not to introduce more than one reactive group into the hirudin molecule. Nevertheless, SPDP-hirudin was on average only 53% as active as unmodified hirudin (17000 ATU) in inhibiting thrombin in the S-2238 assay.

The parent molecules and products of the synthesis of hirudin-59D8 Fab' were analyzed by SDS-PAGE. Under nonreducing conditions, hirudin-59D8 Fab' could be visualized at the anticipated molecular size of about 57 kD. A 1:1 molar ratio of hirudin to 59D8 Fab' was to
be expected because each molecule contained only one reactive sulfhydryl group. The reaction products also contained unconjugated 59D8 Fab', which migrates at 50 kD. 59D8 Fab' could not be separated from the conjugate on the β-peptide-Sepharose affinity column. Under reducing electrophoresis conditions, which cause cleavage of the disulfide bond linking hirudin to 59D8 Fab', the two component chains of 59D8 Fab' could be visualized. Hirudin stained poorly under the conditions used in this study. For the final preparations used in the assays described below, densitometry showed an approximately 1:1 molar ratio of hirudin–59D8 Fab' conjugate to uncoupled 59D8 Fab'. The average (n=20) specific activity of the hirudin–59D8 Fab' preparation was 570±100 ATU/mg protein. Taking into account the approximately 1:1 mixture of hirudin–59D8 Fab' and uncoupled Fab', and the molecular-weight ratio of 7 kD for hirudin versus 50 kD for Fab', we calculated an approximate specific activity of 8595 ATU/mg for coupled hirudin. Thus, it appears that no significant loss in specific activity occurred through the disulfide exchange reaction. (The specific activity of SPDP-modified hirudin was approximately 9010 ATU/mg.)

**Fig 3.** Graphs showing (A) fibrin deposition for hirudin and hirudin–59D8 IgG conjugate on the surface of a clot suspended in fibrinogen solution: hirudin at 80 ATU/mL (○), 8 ATU/mL (●), and 2.4 ATU/mL (△), and hirudin–59D8 IgG at 7.4 ATU/mL (▲) and 1.2 ATU/mL (☆). B, Fibrin deposition on the surface of a clot suspended in fibrinogen solution as a function of thrombin inhibition by hirudin (○) and hirudin–59D8 IgG conjugate (▲) at 120 minutes. SDs in B were estimated from the residual sum of squares yielded by each linear regression, of the form fibrin=a+b·ln(ATU/mL).

**Fig 4.** Graphs showing (A) time course of fibrin deposition for hirudin and hirudin–59D8 Fab' conjugate on the surface of a clot suspended in fibrinogen solution: hirudin at 100 ATU/mL (○), 30 ATU/mL (△), and 10 ATU/mL (△), and hirudin–59D8 Fab' at 9.25 ATU/mL (▲), 3 ATU/mL (●), and 1 ATU/mL (☆). B, Fibrin deposition on the surface of a clot suspended in fibrinogen solution as a function of thrombin inhibition by hirudin (○) and hirudin–59D8 Fab' conjugate (▲) at 180 minutes. SDs in B were estimated from the residual sum of squares yielded by each linear regression, of the form fibrin=a+b·ln(ATU/mL).

**Thrombin Inhibition in the Absence of Fibrin (the Target Molecule)**

The thrombin-inhibitory activity of hirudin was compared with that of hirudin–59D8 IgG conjugate (or hirudin–59D8 Fab' conjugate) in the absence of the target molecule (fibrin) by measuring inhibition of thrombin peptidase activity. Thrombin-dependent cleavage of chromogenic substrate S-2238 was recorded at 405 nm. In subsequent experiments, appropriate dilutions of hirudin, hirudin–59D8 IgG, and hirudin–59D8 Fab' were used so that inhibition of thrombin peptidase activity was identical for each experimental point.

**Thrombin Inhibition as a Function of Hirudin Binding to β-Peptide**

Since hirudin does not bind specifically to the amino terminus of fibrin, we were not surprised by the results in Fig 1A, which show that, at equivalent initial thrombin-inhibitory concentrations, ultimate percent inhibition for the hirudin–59D8 IgG conjugate was 12-fold greater than that for hirudin in an assay that depends on binding to the β-peptide (B1, 55±2% versus 4.3±0.5%,
Inhibition of Thrombus Growth in Fibrinogen Solution

Fig 3 compares fibrin deposition on the surface of a plasma clot suspended in fibrinogen solution in the presence of hirudin with that in the presence of hirudin-IgG conjugate. Because interassay variability is quite large with this test, we made intra-assay comparisons whenever possible. Conjugate at 7.4 ATU/mL was indistinguishable from hirudin at 80 ATU/mL in allowing deposition of fibrin (at a mean of 13 μg less deposition than hirudin) (Fig 3A). Conjugate at 7.4 ATU/mL allowed significantly less fibrin deposition than hirudin at 8 ATU/mL (233 μg less, P = .0002) and hirudin at 2.4 ATU/mL (252 μg less, P = .0001). Conjugate at 1.2 ATU/mL allowed significantly more fibrin deposition than hirudin at 80 ATU/mL (113 μg more, P = .01). The other comparisons were not significant.

When fibrin deposition was plotted against inhibition of thrombin peptidase activity, hirudin–59D8 IgG conjugate was about 10 times more effective than hirudin (Fig 3B). Fibrin deposition at 1.0 ATU/mL [the constant in the linear regression of fibrin deposition versus ln(ÅTU/mL)] for hirudin–59D8 IgG was significantly lower than for unconjugated hirudin (P = .003). The slopes were not different.

Results for hirudin–59D8 Fab’ were similar (Fig 4). Fibrin deposition for hirudin–59D8 Fab’ at 9.25 ATU/mL was 21 μg lower than that for hirudin at 100 ATU/mL (P = .001), 71 μg lower than that for hirudin at 30 ATU/mL (P = .0001), and 129 μg lower than that for hirudin at 10 ATU/mL (P = .0001) (Fig 4A). Also, fibrin deposition for hirudin–59D8 Fab’ at 3 ATU/mL was significantly lower, by 74 μg, than that for hirudin at 10 ATU/mL (P = .01). All other fibrin deposition curves were indistinguishable. For the dose-response curves (Fig 4B), the fibrin deposition at 1.0 ATU/mL for hirudin–59D8 Fab’ was significantly lower than that for unconjugated hirudin (P = .003); the slopes were equal.

Inhibition of Thrombus Growth in Human Plasma In Vitro

We then determined whether the enhanced potency of hirudin–59D8 Fab’ could also be demonstrated in human plasma. Fig 5A shows that fibrin deposition was 84 μg lower for conjugate at 10 ATU/mL than for hirudin at 10 ATU/mL (P = .0001) and 101 μg lower than for the NaCl control (P = .0001), whereas deposition was indistinguishable from that for hirudin at 100 ATU/mL. Fibrin deposition for the conjugate at 1 ATU/mL was 90 μg higher than that for hirudin at 100 ATU/mL (P = .0001) and was indistinguishable from that for hirudin at 10 ATU/mL or that for the NaCl control. Fibrin deposition for hirudin at 100 ATU/mL was 112 μg lower than that for the NaCl control (P = .0001). With a 17-μg lower mean fibrin deposition over time, hirudin at 10 ATU/mL was no different from the NaCl control.

For the dose-response curves (Fig 5B), the fibrin deposition at 1.0 ATU/mL for hirudin–59D8 Fab’ was significantly lower than that for hirudin (P = .0003). These results in plasma were similar to those in fibrinogen solution (compare Figs 4B and 5B); in both systems, the fibrin-targeted hirudin conjugate was 10 times as potent as hirudin.

P < .0001). Hirudin–59D8 Fab’ was as potent as the IgG conjugate (Fig 1B). At 32.5 ± 3.5%, ultimate inhibition by the Fab’ conjugate was significantly greater than that by unconjugated hirudin (0%; P < .0001). This difference indicates that a single antigen combining site is sufficient for effective targeting.

Thrombin Inhibition as a Function of Hirudin Binding to Immobilized Fibrin

Hirudin, hirudin–59D8 IgG, and hirudin–59D8 Fab’ (at equivalent thrombin inhibitory concentrations) were equilibrated with fibrin-Sepharose, and inhibition of thrombin peptidase activity was determined after the Sepharose had been washed. At the highest level of thrombin inhibition, hirudin–59D8 IgG was 1100-fold more potent (P < .0001) and hirudin–59D8 Fab’ was 1900-fold more potent (P < .0001) than unconjugated hirudin (Fig 2). (Because hirudin has not been reported to bind to fibrin, we expected little hirudin activity in this assay.) The Fab’ and IgG conjugates were equipotent on a molar basis. Eight hundred units of hirudin was required to obtain 32.3% thrombin inhibition, in contrast to 1 U hirudin–59D8 IgG, which effected 39.9% inhibition, or 0.5 U hirudin–59D8 Fab’, which effected 37.2% inhibition.
Inhibition of Fibrinopeptide A Generation in Human Plasma In Vitro

To verify that inhibition of thrombus growth was caused by inhibition of thrombin activity, we measured the concentration of fibrinopeptide A in the plasma supernatant of the clot. Fig 6 shows that inhibition of fibrin deposition (Fig 6A) generally paralleled inhibition of the formation of fibrinopeptide A (Fig 6B). When 3 U hirudin was added, there was a significant inhibition of both fibrin deposition and fibrinopeptide A generation in comparison with the saline control. Three units of conjugate resulted in nearly complete inhibition of both fibrin deposition and fibrinopeptide A generation (P<.0001).

Comparison of Hirudin With a Mixture of Hirudin and Antibody

To exclude any influence of uncoupled antibody or Fab' on the activity of hirudin or on the deposition of fibrin, we compared a 1:3 molar mixture of hirudin and hirudin–59D8Fab' with hirudin alone. Fig 7 shows that the mixture of components was not significantly more potent than hirudin alone. The fibrin-deposition curves for hirudin at 44 ATU/mL and the mixture at 43 ATU/mL were indistinguishable. Hirudin at 10 ATU/mL and the mixture at 9 ATU/mL constituted another indistinguishable pair. All inhibitor curves were significantly different from the NaCl control curve (P<.00005). Fibrin deposition for hirudin at 44 ATU/mL was lower than that for the mixture at 9 ATU/mL (P=.003). Deposition for the mixture at 43 ATU/mL was lower than that for hirudin at 10 ATU/mL (P=.01).

Discussion

The fibrin-targeted hirudin molecule is about 57 kD, which corresponds to a 1:1 molar ratio of hirudin and 59D8Fab' in the conjugate. When hirudin and hirudin–59D8Fab' were compared at equivalent thrombin-inhibitory concentrations in the presence of immobilized β-peptide or immobilized fibrin monomer (after nonadherent reactants had been removed by washing), fibrin-targeted hirudin demonstrated a substantial increase in thrombin inhibition. This increase was probably caused by the binding of the antibody component of the conjugate to either free β-peptide or β-peptide at the amino terminus of fibrin monomer. A more realistic test of antibody targeting of hirudin activity is provided by the experiments in which fibrin deposition on preformed clots was examined in a fibrinogen solution and in plasma. In both experiments, fibrin-targeted hirudin was significantly more potent than hirudin in inhibiting fibrin deposition. A decrease in fibrin deposition paralleled a decrease in the generation of fibrinopeptide A, pointing to thrombin inhibition as the mechanism for the increased potency of hirudin–59D8 Fab'. To exclude any interaction between uncoupled antibody and hirudin, we
compared an equimolar mixture of hirudin and 59D8 Fab’ with hirudin alone. The potency of the mixture was not significantly different from that of hirudin.

It is of interest that the epitope recognized by the antibody component of the fibrin-targeted antithrombin is present on fibrin but not on fibrinogen. This epitope becomes available only after thrombin cleaves the β-chain of fibrinogen. Thus, in potential clinical applications, antibody-targeted hirudin is likely to concentrate hirudin activity at sites of thrombosis. Because the hirudin would be concentrated at the thrombus, the plasma concentration of hirudin required for anticoagulation could be lower. As a consequence, it would be possible to anticipate fewer hemorrhagic complications.

If studies in animal models continue to show the promise of these in vitro observations, the construction of a recombinant fusion protein between hirudin and the antibody would be of value. In our previous studies of antibody-plasminogen activator conjugates, the behavior of a specific chemical conjugate often predicted that of a recombinant fusion protein. It follows that fusion proteins could also be constructed from other antithrombins (such as anti-factor Xa) and other thrombus-directed antibodies (such as those to epitopes on the platelet surface). Clinical applications could include any situation in which the therapeutic aim is localized inhibition of thrombosis as contrasted with systemic anticoagulation.

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