Rational Design, Recombinant Preparation, and in Vitro and in Vivo Characterization of Human Prothrombin-derived Hirudin Antagonists*

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A mutant derivative of human prothrombin in which active site aspartate at position 419 is replaced by an asparagine (D419N-prothrombin) has been designed, expressed in recombinant Chinese hamster ovary cells, and purified to homogeneity. D419N-prothrombin was converted to the related molecules D419N-meizothrombin and D419N-thrombin by limited proteolysis by Echis carinatus and Oxyuranus scutellatus venom protease, respectively, and affinity-purified using an immobilized modified C-terminal hirudin-derived peptide. Neither D419N-thrombin nor D419N-meizothrombin exhibited thrombin activity. Titration resulted in no detection of the active site, but binding to the most specific thrombin inhibitor, hirudin, was conserved in both proteins. In vitro examinations showed that D419N-thrombin and D419N-meizothrombin bind to immobilized hirudin, neutralize hirudin in human blood plasma as well as in the purified system, and reactivate the thrombin-hirudin complex. Animal model studies confirmed that D419N-thrombin and D419N-meizothrombin act as hirudin antagonist in blood circulation without detectable effects on the coagulation system. Thus, both D419N-thrombin and D419N-meizothrombin combine for the first time hirudin-neutralizing properties with the advantages of recombinant production of human coagulation factors.

Hirudin, the 65-residue peptide anticoagulant from the salivary gland of the European leech Hirudo medicinalis, is the most specific and most effective inhibitor of the blood protease thrombin (1, 2). The hirudin-thrombin complex has a very low equilibrium dissociation constant in the femtomolar range. Thrombin (coagulation factor II) is the activation product of prothrombin (coagulation factor II) and is a highly potent stimulant of platelets (4). In vivo thrombin is liberated when prothrombin is activated by factor Xa, factor V, and calcium ions (5). Based on pharmacological and clinical profiling of recombinant hirudin, clinical indications such as surgical anticoagulation, medical anticoagulation, adjuvantive administration with thrombolytic agents, anticoagulation during percutaneous transluminal coronary angioplasty, postoperative thrombosis prophylaxis, prevention of rhabdomyosarcoma, microvascular surgery, and anticoagulant in hemodialysis and extracorporeal circulation are explored (6–8).

A main concern in the use of such potent and specific thrombin inhibitors is the risk of bleeding associated with the initial effect of this drug on hemostasis, particularly when the therapy is combined with invasive procedures, fibrinolytic treatment, or the predisposition of the patient to abnormal bleeding (1, 6–10). Thus, availability of an antagonist to hirudin would be essential for instant neutralization of the antithrombotic action. However, such a hirudin antagonist is unavailable at this time. A hirudin-neutralizing agent should exhibit the following properties: 1) it should bind to hirudin as strong as thrombin; 2) the agent should not produce immune or allergic reactions; 3) it should be well tolerated in vivo to be inert to the coagulation and fibrinolytic system.

In this report, we describe the in vitro and in vivo characterization of two derivatives of human prothrombin, D419N-thrombin and D419N-meizothrombin. Neither D419N-thrombin nor D419N-meizothrombin exhibited thrombin activity but did bind and neutralize hirudin in both the purified system and in human plasma. Characterization of D419N-thrombin and D419N-meizothrombin in an animal model showed that both recombinant derivatives neutralized hirudin in the circulation, thus providing evidence on the safe and effective antagonism of hirudin in vivo.

EXPERIMENTAL PROCEDURES

Materials—Materials were purchased from the following companies: Oxyuranus scutellatus venom protease, Sigma; Echis carinatus venom protease and AcOH-HD-CHG-Ala-Arg-pNA, Pentapharm; r-hirudin, Rhein Biotech; partial thromboplastin reagent and coagulation factor II-deficient plasma, IMMUNO AG; NHS-activated Sepharose 4 Fast Flow, Pharmacia Biotech Inc.; microtiter plates (Maxisorb), Nunc; 3,3′,5,5′-tetramethylbenzidine and hydrogen peroxide, Bio-Rad.

Construction of r-prothrombin and D419N-prothrombin Expression Vectors—The expression vectors used in this study essentially are based on pSV2(11). From pSV3β, β-galactosidase insert and polylinker sequence were removed, and a new multiple cloning site was inserted upstream of the intron. Into the resulting plasmid, FII cDNA from pTMemc-PT2, which represents pTM3 (12) with a FII cDNA insert from pTKgpt-PTHBβ (13), was inserted yielding wild-type FII expression vector pSV-FII. The D419N mutant was constructed via polymerase chain reaction. The EcoRV-DraIII fragment from pSV-FII was substi-

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1 The abbreviations used are: D419N-thrombin, recombinant thrombin with Asp419 replaced by Asn; r-prothrombin, recombinant prothrombin; r-thrombin, recombinant thrombin; D419N-prothrombin, recombinant prothrombin with Asp419 replaced by Asn; D419N-meizothrombin, recombinant meizothrombin with Asp419 replaced by Asn; FII, coagulation factor II; r-hirudin, recombinant hirudin; AcOH-HD-CHG-Ala-Arg-pNA, 3-cyclohexylglycyl-alanyl-arginine-p-nitroanilide hydroacetate.
Blood coagulation was initiated by the addition of 100 μl of thromboplastin reagent at 37°C for 3 min. Blood coagulation was determined by mixing 150 μl of mouse citrate-anticoagulated plasma with 50 μl of factor II-deficient plasma and 100 μl of 2 mM CaCl2. Thrombin time was changed to asparagine in the human prothrombin cDNA. Thus, to prepare inactive thrombin but to preserve hirudin interaction with the active center of thrombin (20), Ser925, Asp1193, and His1636 (prothrombin numbering). The activity of thrombin is mediated by the sequential removal of a charge histidine is then stabilized by the negative charged side group of aspartate. Both Ser925 and His1636 contribute to the binding of Ile1 of hirudin at the active center of thrombin (20). To prepare inactive thrombin but to preserve hirudin binding activity, active site aspartate at position 419 was changed to asparagine in the human prothrombin cDNA. D419N-prothrombin was then produced by high cell density perfusion fermentation of recombinant Chinese hamster ovary cells. D419N-prothrombin was purified from cell supernatant by combination of anion exchange chromatography and calcium specific filtration (17). 20 μg of purified D419N-prothrombin was isolated from 1 liter of fermentation supernatant (Fig. 2, lane a). For comparison, r-prothrombin was prepared (Fig. 2, lane b). D419N-prothrombin was then converted to D419N-

**Fig. 1.** Schematic representation of r-prothrombin and D419N-prothrombin expression vectors pSV-FII and pSV-FII D419N. D419N indicates the location of the aspartic acid residue in pSV-FII that has been converted to an asparagine residue in pSV-FII D419N via polymerase chain reaction.

**Fig. 2.** SDS-polyacrylamide gel electrophoresis at reducing conditions of D419N-prothrombin (lane a) and r-prothrombin (lane b). Positions of molecular weight markers are indicated.

**RESULTS**

Preparation of D419N-prothrombin, D419N-meizothrombin, and D419N-thrombin—The x-ray crystal structure of thrombin in its complex with hirudin showed that the COOH-terminal region of hirudin binds to the anion binding exosite of the enzyme, and the N-terminal part of hirudin binds to the thrombin active site (20). The active site of thrombin comprises Ser925, Asp1193, and His1636 (prothrombin numbering). The activity of thrombin is mediated by the sequential removal of a proton from the serine hydroxyl group to histidine. Positive charge histidine is then stabilized by the negative charged side group of aspartate. Both Ser925 and His1636 contribute to the binding of Ile1 of hirudin at the active center of thrombin (20). Thus, to prepare inactive thrombin but to preserve hirudin binding activity, active site aspartate at position 419 was changed to asparagine in the human prothrombin cDNA. D419N-prothrombin was then produced by high cell density perfusion fermentation of recombinant Chinese hamster ovary cells. D419N-prothrombin was purified from cell supernatant by combination of anion exchange chromatography and calcium specific filtration (17). 20 μg of purified D419N-prothrombin was isolated from 1 liter of fermentation supernatant (Fig. 2, lane a). For comparison, r-prothrombin was prepared (Fig. 2, lane b). D419N-prothrombin was then converted to D419N-

**Determination of Coagulation Parameters—**Thrombin activity was determined photometrically by the hydrolysis of the synthetic thrombin-specific chromogenic substrate AcOH-HD-CHG-Ala-Arg-pNA at 37 °C in 50 mM Tris-HCl buffer, pH 8.0, containing 300 mM NaCl, 0.5% albumin, and a final substrate concentration of 200 μM. The release of p-nitroaniline resulting from the hydrolysis of the peptide p-nitroanilide substrate was followed by measuring the increase in absorbance at 410 nm. A thrombin concentration standard was used to prepare the calibration curve.

**Determination of Coagulation Parameters—**Partial thromboplastin time was determined by mixing 50 μl of mouse citrate-anticoagulated plasma with 50 μl of factor II-deficient plasma and 100 μl of partial thromboplastin reagent at 37 °C for 3 min. Blood coagulation was initiated by the addition of 100 μl of 25 mM CaCl2. Thrombin time was determined by mixing 50 μl of mouse citrate-anticoagulated plasma with 150 μl of coagulation factor II-deficient plasma for 1 min at 37 °C. Blood coagulation was initiated by an addition of 100 μl of thrombin standard solution of 7 units ml⁻¹. Determination of D419N-thrombin and D419N-meizothrombin Plasma Concentrations—Plasma concentrations of D419N-thrombin and D419N-meizothrombin were determined by adding serial plasma dilutions to immobilized r-hirudin followed by detection of D419N-thrombin and D419N-meizothrombin with sheep antithrombin IgG peroxidase conjugate (18). Reference curves were prepared with D419N-thrombin and D419N-meizothrombin concentrations ranging from 3 ng ml⁻¹ to 100 ng ml⁻¹. D419N-thrombin/D419N-meizothrombin Binding to Immobilized Hirudin—100 μl of r-hirudin (2 μg ml⁻¹ in 50 mM carbonate buffer, pH 9.6) was coated on microtiter plates at 4 °C for 16 h. Excess unbound hirudin was removed and microtiter plates were washed three times with 10 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Tween 20 (TBS-Tween) to prevent further protein from being adsorbed onto the surface. Individual wells were first incubated with 100 μl of different concentrations of protein for 60 min and then washed three times with TBS-Tween. 100 μl of solution of sheep anti-human thrombin IgG-peroxidase conjugate (1:2000 dilution in TBS-albumin) was applied onto each well, and microtiter plates were incubated at room temperature for 1 h. Excess unbound conjugate was removed by washing three times with TBS-Tween. Then 100 μl of the peroxidase substrate solution (3,3′,5,5′-tetramethylbenzidine and hydrogen peroxide) was added to each well and incubated for 5 min. Peroxidase reaction was stopped by the addition of 100 μl/well of 1 M H2SO4. Color intensity at 450 nm was measured by an automated plate reader (absorbance at 450 nm). Color intensity data were corrected for background readings.

**Electrophoretic Analyses—**Electrophoretic analysis of proteins was performed under reducing conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% acrylamide slab gels using the buffer system described by Laemmli (19).
meizothrombin and D419N-thrombin by limited proteolysis (Fig. 3B). 3 mg of purified D419N-meizothrombin and 1.9 mg of purified D419N-thrombin were obtained from 6 mg of D419N-prothrombin. D419N-thrombin and D419N-meizothrombin were analyzed by N-terminal amino acid sequence analysis. While D419N-thrombin was composed of the thrombin A- and B-chain, D419N-meizothrombin contained the thrombin B-chain and the F1/F2/A-chain. For comparison, r-prothrombin was converted to r-thrombin (Fig. 3A). Both D419N-thrombin, D419N-meizothrombin, and r-thrombin were purified from activation mixtures by affinity chromatography on a newly designed peptide matrix. A synthetic modified derivative of the C-terminal part of hirudin (amino acids 45–64) was covalently bound to Sepharose, yielding an affinity matrix with thrombin anion binding exosite specificity.

**In Vitro Characterization of D419N-thrombin and D419N-meizothrombin—** D419N-thrombin and D419N-meizothrombin were analyzed for thrombin activity. Both D419N-thrombin and D419N-meizothrombin exhibited no enzyme activity toward the thrombin-specific chromogenic peptide substrates AcOH-HD-CHG-Ala-Arg-pNA, while r-thrombin exhibited a specific activity of 98.4 nmol min⁻¹ mg⁻¹ protein. Using p-nitrophenyl-guanidinobenzoate (21), active site titration of r-thrombin resulted in 16.34 nmol mg⁻¹ protein, but no active site was detected for D419N-thrombin and D419N-meizothrombin.

To screen whether D419N-thrombin, D419N-meizothrombin, and r-thrombin exhibit hirudin binding activity, r-hirudin was immobilized at the solid phase of microtiter plates and was incubated with different protein concentrations. D419N-meizothrombin, D419N-thrombin, and r-thrombin bound to immobilized r-hirudin in a concentration-dependent manner (Fig. 4). D419N-prothrombin and r-prothrombin exhibited no hirudin binding activity. Similar to thrombin (18), the binding of D419N-thrombin and D419N-meizothrombin to immobilized r-hirudin was inhibited by the addition of soluble r-hirudin at an equimolar concentration (Fig. 5).

To test whether D419N-meizothrombin and D419N-thrombin neutralize hirudin in a purified system, different concentrations of D419N-thrombin and D419N-meizothrombin were mixed with a constant concentration of r-hirudin. After 1 min of incubation, remaining thrombin activity was determined spectrophotometrically (Fig. 6).
Substrate hydrolysis of a thrombin solution was measured in the spectrophotometric assay. The release of p-nitroaniline was followed over a period of 15 min. r-Hirudin was added after 3 min, and inhibition of thrombin activity was reflected by the slowing of the reaction rate. After another 4 min, D419N-thrombin and D419N-meizothrombin, respectively, were added to the reaction mixtures, and the release of p-nitroaniline was followed for a period of 8 min (Fig. 7).

It appears that the addition of either D419N-thrombin or D419N-meizothrombin to the thrombin-hirudin complex results in a restoration of thrombin activity. Interestingly, it took approximately 2 min for restoration of thrombin activity. Thus, both D419N-thrombin and D419N-meizothrombin bind the r-hirudin that dissociated from the thrombin-hirudin complex. After 2 min the balance between the thrombin-hirudin complex and free thrombin was shifted in favor of free thrombin, with the main portion of hirudin apparently bound to D419N-thrombin and D419N-meizothrombin, respectively.

Neutralization of r-hirudin by D419N-thrombin and D419N-meizothrombin was analyzed in plasma by determination of the partial thromboplastin time. Human citrate-anticoagulated plasma was obtained and analyzed for partial thromboplastin time, thrombin time, and plasma concentration of D419N-thrombin and D419N-meizothrombin, respectively (Table I). The data in Table I show that injection of r-hirudin (test groups 2 and 6) resulted in an increase in partial thromboplastin time of about 75%, an increase in thrombin time of about 60%, and no detection of thrombin in plasma. Injection of D419N-thrombin (test group 3) and D419N-meizothrombin (test group 7) alone resulted in no significant change in coagulation parameters compared to test groups 1 and 5, respectively, but detection of both proteins in mice plasma. Injection of r-hirudin followed by D419N-thrombin (test group 4) and injection of hirudin followed by D419N-meizothrombin (test group 8) resulted in the normalization of partial thromboplastin time and thrombin time. Apparently, both proteins neutral-
ized hirudin in the circulation, thus reducing the free r-hirudin concentration. The hirudin complexed forms of D419N-thrombin and D419N-meizothrombin are less reactive toward immobilized hirudin; thus lower concentrations of D419N-thrombin and D419N-meizothrombin were detected in plasma.

**DISCUSSION**

The development of recombinant hirudin has added a new dimension to the area of therapeutic and surgical anticoagulation (2, 6–10, 22–24). For cautionary reasons in case of bleeding, it would be advantageous to have available a hirudin-neutralizing agent to antagonize instantly the antithrombotic action of hirudin (1, 6–10). Using D419N-prothrombin produced by industrial scale fermentation of recombinant Chinese hamster ovary cells as a starting material, we have prepared, purified, and characterized two derivatives, D419N-meizothrombin and D419N-thrombin. Apart from the point mutation at amino acid position 419 and the cleavage of the peptide chain between Arg<sup>320</sup> and Ile<sup>321</sup>, D419N-meizothrombin corresponds to prothrombin and is composed of the F1/F2-A-chain (Ala<sup>1</sup> to Arg<sup>320</sup>) and the B-chain (Ile<sup>321</sup> to Glu<sup>579</sup>) linked by a single disulfide bond. D419N-thrombin contains the A-chain (Thr<sup>572</sup> to Arg<sup>320</sup>) and the B-chain linked by the same disulfide bond. While human prothrombin is an inactive precursor molecule, human meizothrombin and human thrombin hydrolyze low molecular weight peptidyl substrates. However, only human thrombin is able to convert fibrinogen to fibrin. The molecular basis of the hirudin-thrombin binding includes interactions of the anion binding exosite of the thrombin molecule and the C-terminus of hirudin and interactions of the active-site cleft of the enzyme and the N-terminus of the inhibitor (20). It is commonly thought that the inactivity of meizothrombin toward physiological substrates such as fibrinogen is caused by the presence of the F1/F2-peptide which may alter the enzyme specificity and/or block fibrinogen from binding to the anion binding exosite. However, our results show clearly that both enzymatically inactive prothrombin derivatives D419N-meizothrombin and D419N-thrombin bind the thrombin-specific inhibitor hirudin both in vitro and in vivo. Modification of the active site histidine of human thrombin resulted in a 10<sup>6</sup>-fold reduced affinity for hirudin (25) and active site serine O<sub>y</sub> makes a hydrogen bond with Ile<sup>1</sup> of hirudin (26), thus contributing to the binding of the inhibitor. By contrast, the hirudin binding site is preserved in both D419N-thrombin and D419N-meizothrombin.

Neutralization of hirudin in vitro in a purified system produced similar results for both D419N-thrombin and D419N-meizothrombin (Figs. 6 and 7). By contrast, in vitro in human plasma (Fig. 8) and in vivo in the animal model (Table I) D419N-meizothrombin neutralized hirudin more effectively than D419N-thrombin.

In the animal model both proteins failed to exhibit any effects on the coagulation system. By contrast to any synthetic hirudin antagonist, D419N-thrombin and D419N-meizothrombin are human-like proteins. Since both proteins have been made inactive by an amino acid exchange at the center of the molecule, there is no possibility of regaining activity or liberating any toxic serine protease inhibitor, as might happen if active site inhibited thrombin were used as a hirudin-neutralizing agent. Thus, both D419N-thrombin and D419N-meizothrombin combine for the first time hirudin-neutralizing prop-

![FIG. 8. Neutralization of r-hirudin in human plasma by D419N-thrombin and D419N-meizothrombin.](http://www.jbc.org/)

### TABLE I

| Injection time | Parameter | D419N-thrombin test group | D419N-meizothrombin test group |
|---------------|-----------|---------------------------|-------------------------------|
| 0 min         | Saline    | Saline                    | Saline                        |
| 3 min         | Saline    | D419N-TH                  | D419N-TH                      |
| 6 min         | HP        | HP                        | HP                            |
| Partial thromboplastin time(s) | 23.8 | 42.3 | 24.0 | 26.2 |
| Plasma concentration | 11.4 | 19.8 | 11.6 | 11.7 |
|                | 0 | 0 | 16 | 10 |
|                | Saline    | Saline                    | Saline                        |
|                | Saline    | D419N-MZ                  | D419N-Mz                       |
|                | HP        | HP                        | HP                            |
|                | 22.4 | 38.2 | 21.0 | 21.8 |
|                | 11.6 | 19.3 | 11.2 | 12.0 |

Each test group was composed of 10 mice of 20 g body weight. At t = 0 min 200 µl of r-hirudin solution (0.5 mg kg<sup>-1</sup> of body weight) or 200 µl of saline were injected intravenously. At t = 3 min 200 µl of D419N-thrombin (D419N-TH) solution (2.5 mg kg<sup>-1</sup> of body weight) or 200 µl of D419N-meizothrombin (D419N-MZ) solution (5 mg kg<sup>-1</sup> of body weight) or 200 µl of saline were injected intravenously. At t = 6 min blood was taken from anesthetized mice by heart puncture (HP). Citrate-anticoagulated plasma was analyzed for partial thromboplastin time, thrombin time, and plasma concentration of D419N-thrombin and D419N-meizothrombin. Each measurement was performed in triplicate. Mean values are presented.
erties with the advantages of recombinant production of human blood proteins.

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