Construction and Functional Evaluation of a Single-Chain Antibody Fusion Protein With Fibrin Targeting and Thrombin Inhibition After Activation by Factor Xa

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**Background**—Recombinant technology was used to produce a new anticoagulant that is preferentially localized and active at the site of the clot.

**Methods and Results**—The variable regions of the heavy and light chains of a fibrin-specific antibody were amplified by polymerase chain reaction (PCR) with hybridoma cDNA. To obtain a functional single-chain antibody (scFv), a linker region consisting of (Gly 4 Ser) 3 was introduced by overlap PCR. After the scFv clones were ligated with DNA encoding the pIII protein of the M13 phage, high-affinity clones were selected by 10 rounds of panning on the Bβ15-22 peptide. Hirudin was genetically fused to the C-terminus of the variable region of the light chain. To release the functionally essential N-terminus of hirudin at the site of a blood clot, a factor Xa recognition site was introduced between scFv59D8 and hirudin. The fusion protein was characterized by its size on SDS-PAGE (36 kDa), by Western blotting, by its cleavage into a 29-kDa (single chain alone) and 7-kDa (hirudin) fragment, by its binding to β-peptide, and by thrombin inhibition after Xa cleavage. Finally, the fusion protein inhibited appositional growth of whole blood clots in vitro more efficiently than native hirudin.

**Conclusions**—A fusion protein was constructed that binds to a fibrin-specific epitope and inhibits thrombin after its activation by factor Xa. This recombinant anticoagulant effectively inhibits appositional clot growth in vitro. Its efficient and fast production at low cost should facilitate a large-scale evaluation to determine whether an effective localized antithrombin activity can be achieved without systemic bleeding complications. (Circulation. 2000;101:1158-1164.)

**Key Words:** anticoagulants • antibodies • thrombosis • molecular biology

Inhibition of thrombin by either the indirect thrombin inhibitor heparin or direct thrombin inhibitors such as hirudin reduces thrombus formation after arterial injury in animal models and in humans with unstable coronary syndromes. Furthermore, thrombin inhibitors potentiate fibrinolysis induced by plasminogen activators. Several animal experiments demonstrated that hirudin is more effective than heparin in preventing platelet-dependent arterial thrombosis, rethrombosis after reperfusion, and thrombus growth. However, clinical trials with direct thrombin inhibitors have only been partially successful. High concentrations of hirudin were very effective in inhibiting thrombin but are associated with frequent hemorrhagic complications. A strategy for circumventing this problem is the targeting of hirudin to fibrin.

Fibrin targeting can be achieved with the monoclonal antibody (mAb) 59D8, which selectively binds to the amino-terminus of the fibrin β-chain that becomes exposed after cleavage of fibrinopeptide B by thrombin. Because exposure of this epitope is an early event in the conversion of fibrinogen to fibrin, it is likely that mAb 59D8 accumulates at sites of high thrombin activity, such as a developing arterial clot. Coupling of mAb 59D8 to plasminogen activators resulted in enhanced thrombolytic potency and specificity in vitro and in vivo. A chemical conjugate between hirudin and 59D8 effectively inhibited fibrin deposition on experimental clots and demonstrated potent antithrombotic activity in nonhuman primates. Nevertheless, chemical coupling of hirudin to mAbs has several limitations, the major ones being low yield and loss of hirudin activity. We tried to bypass these limitations by the use of recombinant technology.

Because hirudin needs a free amino- as well as a free carboxy-terminus for antithrombin activity, a direct fusion...
at the termini of hirudin was expected to result in a functional loss. Therefore, a factor Xa cleavage site was introduced between mAb 59D8 and hirudin. This cleavage site was chosen for 2 reasons. First, factor Xa cleaves at the C-terminus of its recognition sequence (Ile-Glu-Gly-Arg) and thus liberates the free amino-terminus of hirudin. Second, factor Xa is a major part of the activated coagulation system at the site of arterial clots and may therefore allow a preferential liberation of functional hirudin at the clot. Without an activated coagulation system, the fusion protein would be inert. However, as a clot develops, the combination of fibrin targeting and dependence on cleavage by factor Xa could result in an effective thrombin inhibition at the clot without systemic anticoagulation.

**Materials and Cells**

Horseradish peroxidase (HRP)–conjugated sheep anti-M13 mAb and mouse anti-E-tag mAb were obtained from Pharmacia, mouse anti-c-myc mAb from Cambridge Research Biochemicals, and goat anti-mouse HRP–conjugated polyclonal antibody (Ab) from Di-anova. BbI5-22, also termed β-peptide, with the amino acid sequence Gly-His-Arg-Pro-Leu-Asp-Lys, was purchased from MWG Biotech. Hirudin was a gift of Knoll AG (Ludwigshafen, Germany). The hybridoma secreting the fibrin-specific mAb 59D8 was generated as described previously,9 and cells were grown on DMEM, 10% fetal calf serum, 2 mmol/L L-glutamine, penicillin (10 IU/mL), and streptomycin (10 μg/mL) (all from Gibco) with 5% CO2 at 37°C.

**Construction of a Functional Single-Chain Antibody**

cDNA of 59D8 hybridoma cells was prepared with mRNA purification columns (oligo-dT) and M-MuLV (both from Pharmacia). Amplification of the antibody variable regions and the insertion of the linker sequence were achieved by polymerase chain reaction (PCR). Primer mixes that contained sequences from conserved regions of the variable regions of the heavy (VH) and light (VL) chains were obtained from Pharmacia. The linker sequence (Gly-Ser), was inserted by the addition of a linker fragment.

**Clone Selection With the M13 Phage System**

The PCR products encoding the functional single-chain antibody fragments (scFv) were cloned into the vector pCANTAB5E (Pharmacia). In this vector, an amber stop codon allows expression of soluble scFv in the nonsuppressor strain HB2151 and display of scFv on the M13 phage surface by fusion to the pIII adsorption protein in the suppressor strain TG1. The supernatant of TG1 clones was used for the following panning procedure: A tissue culture flask with a surface area of 25 cm² was coated with 50 μg/ml BSA and 50 μg/ml anti-mouse IgG antibody and blocked with 2% nonfat dry milk in PBS. The phage-containing supernatant was added and incubated for 2 hours at 37°C. Nonadhering phages were removed by washing 20 times with PBS. A TG1 culture was added to the flask for reinfection with bound phages and incubated for 1 hour at 37°C at 250 rpm. This panning procedure repeated 9 times. Positive clones were tested for phage binding on immobilized β-peptide by use of an HRP–conjugated anti-M13 sheep mAb. The best binding clones with the expected fragment size (750 bp) were used to transform HB2151.

**Cloning of scFv59D8 Into the Expression Vector pHOG21**

DNA of scFv clone 33 was cloned into pHOG2116, mutated at position 6 to glutamine,17 and cloned into pOPE5118 (Figure 2).

**Figure 1.** PCR products for VH, VL, and scFv. Electrophoresis was performed on 1% agarose gel, and PCR products were visualized by ethidium bromide.

**Figure 2.** Maps of pCANTAB5E-scFv59D8, pHOG21-scFv59D8, and pOPE51-scFv59D8-HIR. RAMP indicates ampicillin resistance gene; ColE1 ori, origin of replication of E. coli; M13ori, origin of replication of filamentous phage M13; f1 IG, filamentous intergenic region; Pm/O, lactose regulatable promoter/operator; g3S, signal sequence of pilI; fd3, coding region of pilI gene of M13; pelB, leader peptide sequence of pectate lyases pelB; E, E tag with amino acid sequence GAPVPYPDPLEPR, c-myc, c-myc tag with amino acid sequence EQKLISEEDLN; His6, repeat of 6 histidines; Xa, factor Xa recognition site; and AUsen-STOP, ampicillin stop codon TAG.
Preparation of scFv From Inclusion Bodies

From overnight cultures of XL1-blue, 250 μL was transferred to 5 mL of LB medium containing 100 μg/mL ampicillin and 100 mmol/L glucose and incubated at 37°C and 280 rpm until an OD600nm of 0.8 was reached. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG; 20 μmol/L) and cultured at room temperature for 4 hours. Cells were then centrifuged (6000 rpm, 15 minutes) and resuspended in 165 μL of ice-cold buffer (50 mmol/L Tris-HCl, 100 mmol/L NaCl, 1 mmol/L EDTA, pH 7.0). After freezing and thawing, the sample was centrifuged (12 000g, 4°C, 30 minutes), resuspended in 500 μL of ice-cold TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.4), and incubated for 1 hour at room temperature. Lysozyme (Boehringer Mannheim) was then added to a final concentration of 200 μg/mL, and the incubation was continued for 1 hour, followed by the addition of NaCl (0.5 mol/L) and Triton-X-100 (2.5%) and a final incubation for 1 hour. After centrifugation (12 000g, 4°C, 1 hour), the pellet was washed twice with 3 mol/L urea, and 50 mmol/L ice-cold TE buffer (10 mmol/L Tris-HCl, pH 7.0) and finally solubilized by rotation overnight at 4°C in 250 μL of 6 mol/L GdHCl, 100 mmol/L Tris-HCl, pH 7.0. After centrifugation (12 000g, 4°C, 1 hour), the supernatant was dialyzed against TA buffer (0.4 mol/L arginine-HCl in 100 mmol/L Tris-HCl, pH 7.0).

ELISA With Immobilized β-Peptide

Microwell plates were coated with 1 μg of β-peptide or the control peptide GRGDSP in 100 μL of 0.05 mol/L Na2CO3 (pH 9.6) overnight at 4°C. The plate was then washed 4 times with PBS and blocked with blocking buffer (2% nonfat dry milk in PBS) for 2 hours at room temperature. Samples (100 μL) were incubated for 2 hours at room temperature. After the plate was washed 5 times with PBS, 100 μL of mAb solution (either anti-M13 mAb, anti-c-myc mAb [both 1 to 5000 diluted], or 1 μg/mL anti-E-tag mAb) was added, and after 10 minutes of incubation, the reaction was washed away, and bound scFvs were detected by an anti-E-tag mAb and an HRP-labeled goat anti-mouse Ab.

Affinity Chromatography of scFv and Factor Xa Cleavage

Coupling of β-peptide to sepharose was performed as described previously. Columns containing β-peptide–conjugated sepharose were loaded and washed with TA buffer. Bound protein was eluted by 0.1 mol/L glycine, pH 2.8, and 1 mL fractions were collected and adjusted to pH 7.0 with 0.5 mol/L Tris buffer. Fractions containing significant amounts of product were pooled and dialyzed against TA buffer. For factor Xa cleavage, typically 150 μg of scFv59D8-Xa-hirudin (2 μg/μL) was cleaved by 15 μg of factor Xa (1 μg/μL, Boehringer Mannheim) for various times in 50 mmol/L Tris-HCl, 100 mmol/L NaCl, and 1 mmol/L CaCl2 (pH 8.0) at room temperature.

Measurement of Thrombin Inhibition by scFv59D8-Xa-Hirudin After Factor Xa Cleavage

Inhibition of thrombin was determined by cleavage of the chromogenic substrate S-2238 (Chromogenix). After factor Xa (0.1 μg/μL) cleavage (5 hours, room temperature), 20 μL of thrombin solution (human thrombin, 2.5 U/mL, Sigma) was added to 100 μL of sample in assay buffer (20 mmol/L sodium dihydrosulfinate, 0.15 mol/L NaCl, and 0.1% bovine serum albumin, pH 7.4) and incubated at room temperature for 10 minutes. S-2238 (50 μL, 0.833 mg/mL) was then added, and after 10 minutes of incubation, the reaction was stopped by the addition of 50 μL of 20% acetic acid. Absorbance was measured at 405 nm.

Whole Blood Clot Assay

Except for minor modifications, clot assays were performed as described previously. Clots were initiated by the addition of CaCl2 (16.6 mmol/L) and 2.5 vol% of Actin 7 FS-activated PTT reagent (Dade International) to anticoagulated blood (citric acid, 11 mmol/L) and 2.5 vol% of Actin 7 FS-activated PTT reagent (Dade International) to anticoagulated blood (citric acid, 11 mmol/L) and 2.5 vol% of Actin 7 FS-activated PTT reagent (Dade International) to anticoagulated blood (citric acid, 11 mmol/L) and 2.5 vol% of Actin 7 FS-activated PTT reagent (Dade International) to anticoagulated blood (citric acid, 11 mmol/L). Whole blood was immediately drawn into a silicone tubing (4-mm inner diameter), and clots were allowed to form at 37°C for 1 hour. Quantification of clot size was performed by labeling of blood with125I-fibrinogen (Amersham) at a final activity of 37 500 cpm/mL. The silicon tubing was cut into 1.5-cm fragments, and the formed clots were extruded and washed 5 times in TA buffer. In each assay, the starting size of clots chosen for further experiments varied not more than ±5%. Appositional clot growth was evaluated by the incubation of clots for 10 hours at 37°C on a rotator (60 rpm). Clots were incubated in recalcified citrated whole blood (trace labeled with125I-fibrinogen (Amersham) at a final activity of 37 500 cpm/mL) either with the addition of native hirudin or scFv59D8-Xa-hirudin or without addition. The clots were then washed 10 times with TA buffer, and inhibition of appositional clot growth was evaluated on a γ-counter.

Results

To construct a single-chain antibody directed against fibrin (scFv), mRNA was prepared from 5×10⁷ 59D8 hybridoma cells and reverse transcribed with an oligo-dT primer. The
variable regions of the heavy and light chains (V_H and V_L, respectively) were amplified by polymerase chain reaction (PCR) with primers that anneal to conserved regions at the 5'- and 3'-ends of the variable regions. PCR products of 348 and 339 bp were obtained for V_H and V_L, respectively (Figure 1). After addition of a (Gly 4 Ser) 3 linker by fusion PCR, the scFv product (Figure 1) was cloned into pCANTAB5E (Figure 2) for phage display of the scFv clones. After 10 rounds of panning on immobilized b-peptide, 144 phage clones were tested for binding to the b-peptide by phage ELISA with a horseradish peroxidase (HRP)–conjugated mAb against M13 and by DNA restriction analysis. Twenty-four clones with strong binding to b-peptide and the expected size of the insert were used for transformation of HB2151. The binding properties of soluble scFvs secreted into the supernatant were compared by ELISA with a mouse anti-E-tag mAb and a secondary HRP-conjugated goat anti-mouse antibody (Ab) (Figure 3). Clone 33 demonstrated the best binding properties (Figure 3) and was therefore chosen for sequencing and further characterization. Complementarity determining regions and framework regions of both variable regions and the linker region are highlighted in Figure 4.

For enhanced expression and purification of soluble scFv_59D8, clone 33 was transferred to the expression vector pHOG21 (Figure 2). This plasmid contains a tag sequence coding for 6 histidine residues at the scFv C-terminus, thus facilitating purification by immobilized metal affinity chromatography. However, an additional purification step with ion-exchange chromatography was necessary to obtain a pure product (Figure 5A). An analysis of several eluted fractions by SDS-PAGE is shown in Figure 5B. The functional integrity of the highly purified scFv_59D8 was tested by binding on immobilized b-peptide (Figure 5C). The yield of purified scFv_59D8 was 0.2 mg from 1 L of bacterial culture.

To further increase the yield, glutamic acid at position 6 of the heavy chain was mutated by PCR to glutamine, (Figure 3) and was therefore chosen for sequencing and further characterization. Complementarity determining regions and framework regions of both variable regions and the linker region are highlighted in Figure 4.

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To further increase the yield, glutamic acid at position 6 of the heavy chain was mutated by PCR to glutamine,
because this substitution has been shown to give increased yields of scFvs. Indeed, the yield of functional soluble scFv59D8 was increased \( \approx 4 \) times by this single amino acid substitution.

The factor Xa recognition sequence and the hirudin sequences were fused to the scFv 59D8 by PCR. However, only a low yield of soluble fusion protein was obtained with the pHOG21 expression vector. This was probably due to the high cysteine content (10%) of hirudin that might interfere with the folding process of soluble scFv. To obtain higher levels of the scFv59D8-Xa-hirudin fusion protein, we chose the expression vector pOPE51, which facilitates the production of large amounts of fusion proteins as inclusion bodies in the periplasmic space. When this expression system was used, up to 10 mg of highly purified scFv59D8-Xa-hirudin could be obtained from a bacterial culture of 5 L.

The fusion protein scFv59D8-Xa-hirudin was analyzed by SDS-PAGE and tested for its binding to \( \beta \)-peptide and its susceptibility to factor Xa cleavage. The molecular weight of the intact fusion protein scFv59D8-Xa-hirudin was 36 kDa, that of the cleavage product scFv59D8-Xa was 29 kDa, and that of hirudin was 7 kDa (Figure 6).

We evaluated the functional characteristics of scFv59D8-Xa-hirudin by measuring its binding to \( \beta \)-peptide and by determining its antithrombin activity after binding to \( \beta \)-peptide. Binding to \( \beta \)-peptide was comparable to the binding of equimolar amounts of the Fab fragment of the original mAb 59D8 as measured in ELISA (Figure 7A). The antithrombin activity of the scFv59D8-Xa-hirudin was determined in the presence and absence of factor Xa. ScFv59D8-Xa-hirudin was shown to bind to \( \beta \)-peptide, and the nontarget fusion protein was washed away. The binding function and antithrombin activity of bound scFv59D8-Xa-hirudin could thus be evaluated simultaneously. The uncleaved scFv59D8-Xa-hirudin revealed no antithrombin activity, whereas scFv59D8-Xa-hirudin in the presence of factor Xa demonstrated marked antithrombin activity (Figure 7B).

The ability of the fusion protein to inhibit clot growth was tested in a whole blood clot assay. Native hirudin and scFv59D8-Xa-hirudin were directly compared for their ability to inhibit appositional clot growth. ScFv59D8-Xa-hirudin was able to inhibit clot growth significantly better than native hirudin (Figure 8).
Discussion

Fibrin targeting allows for local enrichment of fibrinolytic agents at the site of the thrombus at low systemic concentrations and thus represents a strategy to increase fibrinolytic potency.10,11 Furthermore, a chemical conjugate of the fibrin-specific mAb 59D8 and the direct thrombin inhibitor hirudin inhibited fibrin deposition on experimental clots12 and demonstrated an increase in antithrombotic potency in baboons.13 To increase the yield and activity of antibody-targeted hirudin and to further improve the risk/benefit ratio of anticoagulation, we have developed a recombinant fusion molecule consisting of an antifibrin single-chain antibody and hirudin. In addition to fibrin targeting, the generation of a free N-terminus, which is essential for the antithrombin activity of hirudin, forms the basis of a unique pharmacological approach. By the addition of the factor Xa recognition sequence, the fusion protein inhibited thrombin only in the presence of factor Xa. Because this factor is part of the activated clotting system and is an important determinant of the procoagulant activity of whole blood clots and arterial thrombi,15 the designed fusion protein represents an anticoagulant that promises to be preferentially active at the site where it is needed.

The potential therapeutic use of single-chain antibody fusion proteins has several major advantages. The variable regions of antibodies comprise the smallest fragments containing a complete antibody binding site, and fusion molecules can be created without loss in binding function of the scFv. Therefore, scFvs are attractive tools for the targeting of drugs, toxins, and radionuclides. The fusion protein scFv59D8-Xa-hirudin with the small molecular size of 36 kDa is expected to be only minimally, if at all, immunogenic, and its small size may improve thrombus accessibility and penetration. It can be produced in bacteria in large amounts, in a short time, and at low cost, and it can be highly purified by affinity chromatography with β-peptide columns, thus providing an ideal situation for drug preparation on a large scale.

Fibrin is an obvious target to concentrate antithrombotic or fibrinolytic agents at the clot. Sufficient amounts of fibrin are present even in platelet-rich thrombi.13 In addition to mAb 59D8, the mAb MA-15C5, directed against the fragment D-dimer of cross-linked human fibrin, has been used successfully to target plasminogen activators to clots.19 Several reports imply that direct thrombin inhibitors may be superior to heparin.3–8 This could be explained by a number of distinct mechanisms. In contrast to heparin, which only inhibits thrombin as a soluble molecule, hirudin can also inhibit thrombin that is bound to the clot or to soluble fibrin degradation products.20,21 Heparin binds to various other partners besides thrombin and is thereby inhibited.22 In contrast to heparin, hirudin has no natural inhibitors.7 Furthermore, hirudin can displace thrombin from platelet thrombin receptors.7 In an experimental study, hirudin but not heparin was even able to dissolve preexisting mural thrombi.7 Nevertheless, the experimental advantages of hirudin compared with heparin have not been reflected by superior clinical performance. Bleeding complications with higher
doses of hirudin appear to be the major limitation.\(^4\) Fusion proteins, such as the one described, provide a promising new development based on the strategy of targeting to and activation at the existing or developing thrombus. This may result in highly efficient inhibition of thrombin and at the same time in fewer bleeding complications.

In summary, a fusion protein has been developed that combines fibrin targeting and antithrombin activity after activation by factor Xa. This recombinant anticoagulant promises to be active only when and where it is needed, thus providing a pharmacological approach that may facilitate an effective anticoagulation without systemic bleeding complications.

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