Thrombin-activable Factor X Re-establishe...
EXPERIMENTAL PROCEDURES

Proteins and Reagents—Human prothrombin and antithrombin were isolated from outdated plasma, thrombin produced through prothrombin conversion, and phospholipid vesicles prepared, as previously described (18–20). Human FIXa, FXa, FXa, and FVIIa, bovine FV/FVa, FX activator isolated from the venom of Russell’s viper (RVV-X), and hirudin (Refludan) from Hoechst (Frankfurt, Germany), and recombinant TF from Dade Behring (La Défense, France) were purchased from Sigma-Aldrich. H-D-Phe-Pip-Arg-CH2Cl (PPACK2) from VWR International (Fontenay-sous-Bois, France); Gly-Pro-Arg-Pro-amide peptide, Phenylmethylsulfonyl fluoride (PMSF), Polybrene, and bovine serum albumin (BSA, protease-free) were purchased from Sigma-Aldrich. H-D-Phe-Pip-Arg-pNA (S2238), Z-D-Arg-Gly-Arg-pNA (S2765), and benzoyl-Ile-Glu(γ-OR)-Gly-Arg-pNA (S2222) were obtained from Biogenic (Maurin, France); H-D-Phe-Phe-Arg-CH2Cl (PPACK2) from VWR International (Fontenay-sous-Bois, France).

Preparation of Recombinant Human FX and Derivatives—The shuttle vector pNUT-FX (21) carrying the cDNA coding for the propeptide of human prothrombin in place of that of FX and with amino acids DQVDPRLIDGK (recognized by monoclonal antibody HPC-4 (Roche Applied Science) at the 3′-end) was used as a template to prepare the vector expressing FXFpA. Constructions were prepared through a single polymerase chain reaction, and sequence coding for FXFpA was verified by dideoxy chain termination, as previously described (21). Platelet Factor 3 reagent (PF3) was purchased from Organon Teknika. Gly-Pro-Arg-Pro-amide peptide, Phenylmethylsulfonyl fluoride (PMSF), Polybrene, and bovine serum albumin (BSA, protease-free) were purchased from Sigma-Aldrich. H-D-Phe-Pip-Arg-pNA (S2238), Z-D-Arg-Gly-Arg-pNA (S2765), and benzoyl-Ile-Glu(γ-OR)-Gly-Arg-pNA (S2222) were obtained from Biogenic (Maurin, France); H-D-Phe-Phe-Arg-CH2Cl (PPACK2) from VWR International (Fontenay-sous-Bois, France).

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Following dilution of 1:10 (v/v) in water, the buffer was exchanged to Tris (50 mM, pH 7.5) containing 500 mM NaCl by anion exchange chromatography onto a Q-Sepharose Fast Flow column (Amersham Biosciences). Calcium concentration was adjusted to 5 mM, and recombinant FX was purified by affinity chromatography as previously described (21). Concentrations of recombinant FX and FXFpA were initially estimated from the absorbance at 280 nm assuming an extinction coefficient of 1.25 mg cm−1 and a molecular weight of 59,000 and 52,400 for FX and FXFpA, respectively (taking into account the FpA and C-terminal epitope sequences). Active zymogen concentrations were later refined by comparing the expected amount of FXa (from A280) with the effective activity obtained after full activation by RVV-X. Prior to use as a zymogen, FX and FXFpA were incubated with 1 mM PMSF to neutralize any trace of FXa. Control experiments indicated that after a 30-min incubation in Tris-HCl buffer, PMSF was fully hydrolyzed and did not interfere with other reactions. Alternatively, possible traces of activated FXFpA were neutralized by twice adding 5 μM PPACK2, each followed by a 30-min incubation at 25 °C. Excess PPACK2 was removed by precipitation at 50% saturation (NH4)2SO4 (2 h, 4 °C); the pellet was resuspended in one-half of the initial volume of kinetic buffer and used immediately. Following treatment, FXa (if any) was below the detection limit (1 pM).

The amount of activable FXFpA recovered was estimated retrospectively by a 12-h incubation of an aliquot with 5 nM RVV-X; concentration of FXa was then deduced from the rate of 200 μM S2765 hydrolysis. N-terminal sequence analysis was performed after transfer onto a polyvinylidene difluoride membrane (Laboratoire de Microsequençage des Protéines, Institut Pasteur, Paris, France).

FX and FXFpA Activation Kinetics—Activation of FX (derived from plasma or recombinant) and of FXFpA was studied at 25 °C, in kinetic buffer (50 mM Tris–HCl, pH 7.5 containing 0.15 M NaCl, 5 mM CaCl2, 0.2% (w/v) poly(ethylene glycol) M, 6000, and 1 mg/ml BSA). Activation of 50 nM FX or FXFpA was triggered by adding 2 nM thrombin, 100 pM FVIIa plus 1.3 nM TF, or 10 nM RVV-X. At timed intervals, an aliquot was mixed with 1 μM antithrombin and 10 μM PPACK2. Traces of FXa were measured by the thrombin generation assay using chromogenic substrate S2238, with data normalized to FX or FXFpA activity in the absence of thrombin. For FXFpA, 50 nM FXa was added to standardize the assay, and 500 nM FXFpA was used to saturate thrombin.

FIGURE 2. SDS-PAGE of FXFpA FX, and activation products. Samples (2–5 μg of protein) were analyzed following disulfide bond reduction. Lane 1, molecular mass markers (as indicated); lane 2, purified FX; lane 3, activated FX; lane 4, wild-type (plasma-derived) human FX; lane 5, wild-type (plasma-derived) human FXa. Bands were visualized by Coomassie Blue staining. Part of FXFpA was expressed as a single chain species migrating with an apparent molecular mass slightly higher than the heavy chain of FX. Consistent with the absence of the activation peptide, the heavy chain of FXFpA migrated well below that of wild-type FX (HC). Following activation, the apparent molecular weight for the heavy (HCα) and light chains (LC) of the activation products from FX and FXFpA, was indistinguishable.
was withdrawn, and the reaction was quenched by adding 200 nM hirudin (activation by thrombin) or 50 mM EDTA (activation by RVV-X and FVIIa). The progress of FXa and activated FXFpA formation was monitored by measuring rates of 200 μM S2765 hydrolysis. Pseudo-first-order rate constants (k) were estimated by non-linear regression analysis of the FXa activity with time using a one-phase exponential association equation. Values of k/Km were deduced from the ratio of k over the activator concentration (thrombin, TF/FVIIa, or RVV-X). Large quantities of FXa were prepared by passage through a HiTrap N-hydroxysuccinimide-activated column (Amersham Biosciences) coupled to RVV-X, as previously described (23). Large quantities of activated FXFpA were prepared by passage through a 1 ml HiTrap N-hydroxysuccinimide-activated column coupled to thrombin (1 mg/ml of gel) according to the manufacturer’s instructions. 4 mg of zymogen in kinetic buffer without BSA were continuously passed through the column at 0.5 ml/min for 16 h at 25 °C. Following activation, FXa and the activated form of FXFpA were purified by chromatography on a heparin-Sepharose column (Amersham Biosciences), and their active site content determined by titration with PPACK2 as previously described (23).

Prior to use, dilution of FXa and activated FXFpA were systematically controlled by measuring the rate of 200 μM S2765 hydrolysis. Values of k/Km of S2222 and S2765 for FXa and activated FXFpA were estimated by standard methods, as previously described (19, 26).

**FXa and Activated FXFpA Inhibition Kinetics**—The overall rate of association (k) for the inhibition of FXa or the activated form of FXFpA by PPACK2 was estimated for FXa at 25 °C as previously described (20, 26). Progress of inhibition by antithrombin was studied at 25 °C in kinetic buffer containing one unit/ml heparin or 5 μg/ml Polybrene. Reactions, carried out in microplates in a volume of 200 μl, were initiated by adding 0.5 mM FXa or activated FXFpA to a mixture of antithrombin and heparin or Polybrene. Antithrombin concentration (3 and 6 mM in the presence of heparin; 0.5 and 1.0 mM with Polybrene) was such that significant inhibition of the enzyme occurred over the time of experiment (less than 20 min). The substrate concentration (S2765; 200 μM) was such that less than 10% was hydrolyzed within 20 min without inhibitor. Observed rates of inhibition (kobs) were estimated by non-linear regression analysis of A405 versus time using Equation 1 for slow-binding inhibition as previously described (20, 26).

\[ A_{405}(t) = v_i t + (v_f - v_i)(1 - \exp(-k_{obs}t))/k_{obs} + A_{405}(0) \]  

(Eq. 1)

in which A405(0) and A405 represent A405 initial (before addition of FXa) and at time t, respectively; v_i and v_f the initial and final rates of S2765 hydrolysis, respectively (v_f is normally zero with serpins). The value of k, was deduced from kobs using Equation 2 to account for the competition introduced by the substrate,

\[ k_a = k_{obs} (1 + S_0/K_m)/I_0 \]  

(Eq. 2)

in which S_0 is the initial S2765 concentration, K_m its Michaelis constant for FXa (or activated form of FXFpA) and I_0 the active concentration of added serpin, estimated as previously described (21).

**Plasma (ex Vivo) Studies of FXa and Activated FXFpA**—The half-life in citrated human plasma was estimated by adding 20% (v/v) kinetic buffer containing 40 mM CaCl_2, 400 units/ml hirudin, and 50 mM FXa (or the activated form of FXFpA). At timed intervals, aliquots were withdrawn and residual activity estimated from the rate of hydrolysis of 200 μM S2765. Observed rates of inhibition (kobs) were estimated by non-linear regression analysis of the residual FXa activity with time using a first-order decay equation. Half-life values were calculated by dividing ln(2) by kobs (21). The anti-hemophilic potential of FXFpA was evaluated by its ability to shorten clotting time and increase thrombin formation in normal FVIII- and FIX-deficient plasmas. Particular care was taken to verify that FXa or activated FXFpA was undetectable prior to addition of their zymogen to plasma. The progress of clot formation was recorded at 37 °C by turbidity measurement. 100 μl of prewarmed plasma were added to a mixture of 10 μl of F3 and 90 μl of kinetic buffer containing 40 mM CaCl_2, and various amounts of either FXFpA, FX, FVIIa, FVIII, or FIX in a prewarmed microplate. A405 was recorded every second, and time required to reach half-maximal turbidity was taken as representative of the clotting time. The procoagulant potential of FX and FXFpA was also evaluated by its ability to induce thrombin formation in normal, FVIII- or FIX-depleted plasma. Clotting was triggered at 37 °C by adding 50% (v/v) kinetic buffer containing 40 mM CaCl_2, 160 μM phospholipid vesicles, 0.5 mM TF, and various amounts of FVIIa, FVIII, FIX, FX, or FXFpA to prewarmed plasma containing 16 mM Gly-Pro-Arg-Pro-amide to prevent fibrin polymerization (27). At timed intervals, aliquots were withdrawn, and reactions quenched by diluting with 1 volume of 1:10 Tris, 50 mM; NaCl, 150 mM; pH 8.0 containing 10 mM EDTA, and 100 mM benzamidine. The amount of thrombin was monitored from the rate of 100 μM S2238 hydrolysis after further dilution (1:20; v/v) in kinetic buffer without BSA (21).

**RESULTS**

**Thrombin Activates FXFpA**—Thrombin specificity originates in part from amino acids framing the arginine preceding the bond cleaved...
within substrates (18, 23), but complementary secondary sites also contribute critically to binding of the major ligands, including fibrinogen (28–30). In conjunction with the 60-loop insertion of thrombin, the argyl binding site provides a remarkable niche for FpA of fibrinogen (22, 28, 31–34). To test if simple substitution for FpA of the activation peptide of FX would permit thrombin cleavage, we prepared a chimera (FXFpA) in which amino acids DFLAEGGGVR replaced the entire activation domain of FX rendered FX activable by thrombin, even if the activation site concentration was determined. Following SDS-PAGE analysis, the yield of thrombin-activated FXFpA was determined. Large quantities of the activated form of FXFpA were produced by thrombin cleavage. Large quantities of FXFpA were produced by passage of FX through a column linked with linear regression analysis using the Michaelis-Menten equation (values of 5.9 ± 0.3 × 10^{-3} s^{-1} for kcat and 1.4 ± 0.2 μM for Km). Table 1 shows the kinetic constants (kcat and Km) for substrate hydrolysis were estimated by non-linear regression analysis of the dependence of the initial rate of cleavage upon substrate concentration using the Michaelis-Menten equation and are given with the S.E.

**Table 1**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>S2222</th>
<th>S2765</th>
</tr>
</thead>
<tbody>
<tr>
<td>kcat</td>
<td>159 ± 8.7</td>
<td>166 ± 5.1</td>
</tr>
<tr>
<td>Km</td>
<td>260 ± 35.2</td>
<td>225 ± 12.5</td>
</tr>
</tbody>
</table>

Activated FXFpA was designed to release normal FXa upon cleavage by thrombin given that FpA would be freed after activation. To verify this assumption, we compared FXa obtained through FX activation by RVV-X with activated FXFpA produced by thrombin cleavage. Large quantities of FXa were prepared by passage of FX through a column linked with RVV-X and large quantities of the activated form of FXFpA were produced by passage of FXFpA through a column linked with thrombin. Activation products were concentrated by heparin-Sepharose chromatography, and their activation products from FX and FXFpA were indistinguishable (Fig. 2). Detection of the activation domain itself, processing and folding seems critical for FX activation. In favor of a limited function, none of the 41 deleterious mutations reported for FX are located in its activation domain (40).

**Thrombin-activable Factor X**

**Table 2**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>kcat</th>
<th>Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>FXa</td>
<td>2.3 ± 0.02 × 10^{-1}</td>
<td>2.3 ± 0.03 × 10^{-1}</td>
</tr>
<tr>
<td>Activated FXFpA</td>
<td>2.2 ± 0.07 × 10^{-1}</td>
<td>1.2 ± 0.15 × 10^{-1}</td>
</tr>
</tbody>
</table>

* Data obtained for inhibition by PPACK2 or antithrombin in the presence of Polybrene were analyzed by non-linear regression using a first-order decay equation.

* Data obtained for inhibition by antithrombin in the presence of heparin were analyzed by non-linear regression using Equations 1 and 2.

**FIGURE 4.** Michaelis-Menten kinetics of FXFpA activation by thrombin. Initial velocities of FXFpA activation by 1 nm thrombin are shown. The curve was obtained by non-linear regression analysis using the Michaelis-Menten equation (values of 5.9 ± 0.3 × 10^{-3} s^{-1} for kcat and 1.4 ± 0.2 μM for Km).

**FIGURE 5.** Inhibition of thrombin-activated FXFpA by antithrombin in the presence of heparin. The graph represents progress curves of 200 μM S2765 hydrolysis by 0.5 nm activated FXFpA alone (open squares), or in the presence of one unit/ml heparin and 3 nM (closed squares) or 6 nm (closed circles) antithrombin. Curves were obtained by non-linear regression analysis using Equation 1 to yield observed rate constants of inhibition (kobs), which were used to calculate kcat according to Equation 2. An average value of 0.12 ± 10^{-3} M^{-1} s^{-1} was obtained for kcat. An estimate of kcat/Km was obtained under the same conditions for FXa inactivation.

**TABLE ONE**

Kinetics of S2222 and S2765 hydrolysis by FXa or activated FXFpA

The kinetic constants (kcat and Km) for substrate hydrolysis were estimated by non-linear regression analysis of the dependence of the initial rate of cleavage upon substrate concentration using the Michaelis-Menten equation and are given with the S.E.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>kcat</th>
<th>Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2222</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>S2765</td>
<td>2.3</td>
<td>2.3</td>
</tr>
</tbody>
</table>

**TABLE TWO**

Kinetics of FXas and activated FXFpA inhibition by PPACK2 and antithrombin

The overall rates of association (kcat) were estimated in the presence of at least two concentrations of inhibitor by progress curve kinetics of substrate hydrolysis and are given with the S.E.

<table>
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<tr>
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<th>Km</th>
</tr>
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* Data obtained for inhibition by PPACK2 or antithrombin in the presence of Polybrene were analyzed by non-linear regression using a first-order decay equation.

* Data obtained for inhibition by antithrombin in the presence of heparin were analyzed by non-linear regression using Equations 1 and 2.
TWO). We also verified that FXα produced through FX or FXFpA activation were neutralized by antithrombin with comparable $k_a$ values (Fig. 5). Finally, the half-life in the plasma of FXα produced through FX or FXFpA activation were similar suggesting again full interchangeability between the two molecules (Fig. 6).

$FX_{FpA}$ Corrects Clotting Time of FVIII- or FIX-deficient Plasmas—

One of the difficulties we encountered in testing anti-hemophilic potential of our thrombin-activable FX was that even traces of FXα shorten the clotting time of FVIII- or FIX-deficient plasmas. As little as 15 pM FXα appreciably decreased the time to clot formation, whereas 250 pM FXα reduced the clotting time to a value comparable to that obtained with normal plasma. Adding FXα to normal plasma also shortened its clotting time (Fig. 7), allowing detection of minute FXα contamination. Thus, it was of utmost importance to ascertain that our $FX_{FpA}$ preparation was exclusively in a zymogen form and devoid of FXα contamination. To this aim, $FX_{FpA}$ was treated twice for 30 min with PPACK2 and separated from excess PPACK2 by (NH₄)₂SO₄ precipitation. Amidolytic activity of PPACK2-treated $FX_{FpA}$ was undetectable. $FX_{FpA}$ prepared as above had no detectable influence on the clotting time of normal plasma, even at concentrations above 100 nM, suggesting that FXα contamination, if any, represented less than 0.01% $FX_{FpA}$ and that excess PPACK2 was properly removed by (NH₄)₂SO₄ precipitation (Fig. 7). This observation also suggested that $FX_{FpA}$ (as a zymogen) did not interfere with coagulation cascade in normal plasma. This is in contrast to FVIIa and FVIII, which under the same conditions shorten the clotting time when added to normal plasma (Fig. 7). Interestingly, wild-type FX also slightly decreased the clotting time of normal plasma. Thus $FX_{FpA}$ could be devoid of an overdose effect, a considerable advantage for handling hemophilia. We then compared the effect of increasing amounts of $FX_{FpA}$ on the clotting time of FVIII- or FIX-deficient plasmas. Adding 64–128 nM $FX_{FpA}$ was sufficient to decrease clotting time to a value comparable to that obtained with normal plasma (Fig. 8). Addition of 64–128 nM FVIIa also corrected the clotting time of FVIII- or FIX-deficient plasmas, as well as 50–100 milliunits of FVIII or 6–12 nM FIX, respectively (Fig. 8). It is to mention, however, that adding wild-type FX also shorten the clotting time of FVIII-depleted plasma, albeit not as efficiently as $FX_{FpA}$ (amount of 450 nM were required to

**FIGURE 6.** Half-life of FXα and thrombin-activated $FX_{FpA}$ in plasma. Remaining activity (%) versus time when 10 nM activated $FX_{FpA}$ (closed squares) or FXα (closed circles) were incubated in normal plasma rendered non-coagulable by addition of hirudin. Curves were obtained by non-linear regression analysis using a one-phase exponential decay equation to yield observed half-life values of 53 ± 6 s and 43 ± 3 s for thrombin-activated $FX_{FpA}$ and FXα, respectively. Probably because of α2-macroglobulin sequestration, which does not prevent amidolytic activity of FXα, inhibition appears incomplete.

**FIGURE 7.** Clot formation in normal plasma. Graphs represent progress curves of clot formation detected from turbidity increase at A₄05. In each panel, open circles (in gray) represent the progression obtained when clotting was triggered at 37 °C by addition of a mixture of PF3 and calcium only. Data points with connecting lines were obtained when clotting was triggered as before, but with increasing amount (from right to left) FXα (15.6, 31, 62.5, 125, 250 500 and 1000 pM), FVIII (0.0156, 0.031, 0.0625, 0.125, 0.25, 0.5, and 1 unit/ml), FVIIa (2, 4, 8, 16, 32, 64, and 128 nM), or $FX_{FpA}$ (2, 4, 8, 16, 32, 64, and 128 nM). Clot formation occurred when half-maximal turbidity (inflection point) had been reached. As little as 15 pM FXα shortened the time to clot, whereas 128 nM $FX_{FpA}$ had no detectable influence.
apparently correct clotting time). Unfortunately, rather than really informative, these observations simply point out that clotting time assays are insufficient to test the potential of anti-hemophilic factors. That about 100 nM FXFpA was sufficient to correct clotting time in FVIII- or FIX-deficient plasmas was surprising at first, because the $k_{cat}/K_m$ value estimated above suggested that, within 3 min, only about 60 pM FXa would be produced through FXFpA activation by 1 nM thrombin. Nevertheless, as already mentioned, addition of 250 pM FXa was sufficient to decrease clotting time of FVIII- or FIX-deficient plasmas to that of a normal plasma. Thus, considering that production of FXa also originated from FX (and FXFpA) activation by the TF-FVIIa complex, it is conceivable that sufficient amount of FXa was produced to correct clotting time (41, 42).

FXFpA Amplifies Thrombin Production in FVIII- or FIX-deficient Plasmas—The clotting time of plasma mimicking that of hemophiliacs suggested that in the presence of FXFpA, thrombin was produced in a quantity allowing enough fibrin to be released for clot formation. As little as 10 nM thrombin is sufficient to induce clotting within 3 min in FVIII-depleted plasma. We therefore wondered what amount of thrombin was actually produced through FXFpA activation once the clotting cascade was triggered. To this aim, Gly-Pro-Arg-Pro-amide was added to FVIII-depleted plasma to prevent fibrin polymerization (27, 43). Use of Ancrod to defibrinate plasma was proscribed, because in addition to hydrolyzing fibrinogen, the snake venom activated rapidly FXFpA carrying FpA in place of its normal activation peptide (data not shown). Thrombin production was triggered in noncoagulable plasmas by adding TF and phospholipid, and aliquots were withdrawn at timed intervals. Activation reactions were quenched and the amount of thrombin estimated through S2238 hydrolysis. Undoubtedly, FXFpA had the potential to correct thrombin generation of FVIII-depleted or FIX-depleted plasmas (Fig. 9). When 128 nM FXFpA was added, the lag phase and maximum amount of thrombin favorably compared with the amount produced when deficient plasma was supplemented with its missing factor. Therapeutic doses of FVIIa, allowing handling of severe hemophilia, result in a plasma concentration of about 70 nM (44). Thrombin production of plasma mimicking that of hemophiliacs also improved dramatically when the clotting cascade was triggered in the

![Figure 8. Clot formation in FVIII-depleted plasma.](http://www.jbc.org/)

![Figure 9. Amplification of thrombin production in FVIII-depleted plasma.](http://www.jbc.org/)
presence of 70 nm FVIIa, yet the total amount remained below that observed in normal plasma. Finally, whereas wild-type FX shortsens clotting time of FVIII-depleted plasma, it influenced thrombin production very little (Fig. 9).

DISCUSSION

In designing a thrombin-activable FX chimera as an antihemophilic factor, we anticipated several decisive advantages. First, reciprocal activation between thrombin/FX_FpA and Fxas/prothrombin would re-establish a true auto-amplification of thrombin production. The normal clotting cascade generates more thrombin than is apparently necessary (3), but a minimum amplification is nevertheless required. The rate of thrombin production should locally exceed that of its neutralization by antithrombin to attain rapidly (less than 3 min) a threshold allowing sufficient fibrin production. Because of the conjunction of several mechanisms acting in synergy, supraphysiologic amounts of FVIIa allow appropriate thrombin production in intrinsic tenase-deficient plasmas (45–49). The lack of cyclic amplification (or positive feedback) implies that FXa production is a linear function of the amount of FVIIa available. Thus restoring hemostasis in deficient plasmas necessitates high doses of FVIIa, yet thrombin production remains below normal (44, 49). Restoring thrombin production with FX_FpA follows a different strategy. Initial triggering of the clotting cascade is functional in hemophiliacs, only amplification is defective. The FX_FpA cascade would rely on the same mechanisms for triggering, but would re-establish an intrinsic amplification of the signal. Initial traces of thrombin would slowly transform FX_FpA into Fxas, which in turn would cause more prothrombin conversion and subsequently more FX_FpA activation. Thus, following an initial triggering, thrombin production through FX_FpA activation would auto-amplify, once a threshold had been reached. Such cyclic amplification comprises a potential for exponential enhancement, as occurs in normal clotting cascade through the intrinsic tenase complex. A second likely advantage of the FX_FpA approach would be little immunogenicity. Severe A-type hemophiliacs resist FVIII therapy because they develop antibodies against what they identify as a foreign protein. FVIIa therapy is exempt from this complication because FVIIa is normally present in hemophilic blood. Similarly, FX_FpA could be appropriate to treat hemophiliacs developing inhibitors, because FX and FpA would be normally present in treated hemophilic blood. A third benefit of FX_FpA could be its plasma half-life. Based on that of normal FX, the half-life of the FX chimera could be as long as 30 h (50), far longer than that of FVIIa (less than 3 h). Finally, and perhaps most importantly, activation and neutralization of FX_FpA appeared self-controlled. FX_FpA activation would occur only in the presence of thrombin (or TF-FVIIa complex), thus would be confined to the site of injury, where a large amount of thrombin is needed. Systemic activation would be constrained by the relatively slow rate of FX_FpA activation by thrombin, thus limiting the risk of uncontrolled initiation. Through the intrinsic tenase complex, normal hemostasis triggers FXa production several 100-fold more efficiently, yet handles prevention of FXa and thrombin dissemination through neutralization by antithrombin. It is unlikely that introducing a comparatively slow amplification loop could saturate mechanisms that normally confine the clot at the site of injury. Consistent with this hypothesis, adding the chimera to normal plasma had no detectable effect on clot formation, contrary to therapeutic doses of FVIIa or FVIII, which both shortened the time to clot formation.

Hrachcivonina et al. (17) proposed an elegant approach based on an increase of circulating procoagulant microparticles to correct hemostasis in hemophiliacs. As with FVIIa-based therapy, however, there is no cyclic amplification of thrombin production. In addition, infusion of a soluble chimera of P-selectin reduces the clotting time of wild-type mice, this lacks self-control. The combination of FXa and phospholipid vesicles (13, 14) as well as acylated-FXa, which slowly hydrolyzes to FXa in blood (15, 16), are other therapies that effectively bypass the intrinsic tenase complex in vivo. Again, these approaches do not induce cyclic amplification of thrombin generation, and, as above, potential drawbacks result from the difficulty in controlling FXa generation, occurring slowly but continuously, out of control, and without localization at the site of injury. Finally, Kwan et al. (51) described a self-activating FX derivative in which the activation sequence was changed to that of prothrombin, and Rudolph et al. (39) prepared several variants of FX that also auto-activate. Theoretically, these FX derivatives must permit an auto-amplification of FXa production equivalent to that obtained with FX_FpA. These FX derivatives have not been designed or tested for the purpose of hemophiliac treatment. Whether adding self-activating FX in intrinsic tenase-deficient plasma safely corrects hemostasis should be investigated in future studies.

REFERENCES

Thrombin-activable Factor X Re-establishes an Intrinsic Amplification in Tenase-deficient Plasmas

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