Quantitation of Activated Factor VII Levels in Plasma Using a Tissue Factor Mutant Selectively Deficient in Promoting Factor VII Activation

By James H. Morrissey, B. Gail Macik, Pierre F. Neuenschwander, and Philip C. Comp

Although the majority of factor VII (FVII) circulates in the zymogen form, low levels of activated factor VII (FVIIa) have been postulated to exist in plasma and to serve a priming function for triggering of the clotting cascade. However, direct measurement of plasma FVIIa has not previously been possible. We have quantified plasma FVIIa levels using a novel, highly sensitive assay that is free from interference by FVII. Specificity of this clot-based assay results from the use of a mutant tissue factor that is selectively deficient in promoting FVII activation, but retains FVIIa cofactor function. In normal adults, FVIIa was found to be present in plasma (mean: 3.6 ng/mL) with considerable variation between individuals (range: 0.5 to 8.4 ng/mL). FVIIa levels were only loosely correlated with FVII coagulant activity, but were elevated in pregnancy and reduced with oral anticoagulant therapy. Incubation of plasma on ice in glass containers (cold activation) resulted in substantial FVIIa generation. Measurement of plasma forms of factor VII is of potential clinical importance because elevated FVII coagulant activity has been implicated as a significant risk predictor for ischemic heart disease. Clinically, this new assay will now permit direct assessment of the role of plasma FVIIa in thrombotic disorders.

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Therefore, direct measurement of plasma FVIIa itself is necessary to assess the degree of FVII activation. The reason that existing assays of FVII:C suffer from interference from zymogen FVII is that they initiate clotting with thromboplastin (the active ingredient of which is TF), and TF strongly promotes the conversion of FVII to FVIIa. A clotting assay specific for FVIIa—and free of interference by zymogen FVII—could theoretically be devised if TF were altered so that it retained cofactor function toward FVIIa but no longer supported conversion of FVII to FVIIa. We now report the development of such a clotting assay specific for FVIIa and apparently free of interference from zymogen FVII. This simple assay uses a soluble mutant form of TF, which we recently demonstrated has selectively lost the ability to promote FVII activation, while at the same time retaining function as a cofactor for FVIIa-catalyzed activation of factor X.9 The assay permits direct measurement of FVIIa in plasma, and should facilitate investigation into the relationship between plasma FVIIa levels and thromboembolic disease. It also provides a sensitive new way of monitoring the ongoing activation or turnover of the clotting system. In addition, the new assay should provide a rapid, quantitative means of monitoring plasma FVIIa levels in patients undergoing oral anticoagulant therapy or receiving recombinant FVIIa therapy.22

MATERIALS AND METHODS

Reagents. Recombinant human FVIIa was purchased from NovoBiolabs (Danbury, CT). Human plasmas congenitally deficient in FVII or factor IX were purchased from George King Bio-Medical (Overland Park, KS). Immuno-depleted FVII-deficient human plasma was purchased from Baxter Diagnostics (Miami, FL). These deficient plasmas had less than 1% residual factor activity, according to the suppliers. Rabbit brain cephalin and mixed rabbit brain phospholipids (KCI-washed, chloroform:methanol extract of rabbit brain) were purchased from Sigma Chemical Co (St Louis, MO). Purified phosphatidylcholine and phosphatidylserine were purchased from Avanti Polar Lipids (Pelham, AL).

Protein purification. Wild-type FVII was purified from human plasma and activated to FVIIa as described.4 Recombinant human FVII was expressed and purified as described.4 Recombinant, soluble human TF (sTF25-39) was expressed in Escherichia coli and purified as described elsewhere.23 Bacterially expressed sTF25-39 consists only of the extracellular domain of TF and is comparable with recombinant sTF25-39 produced in mammalian cells.24 Soluble TF produced in either mammalian cells or bacteria was found to perform identically in the new soluble TF clotting assay for FVIIa described below (data not shown). Concentrations of purified sTF25-39 were determined spectrophotometrically using an E210 of 14.82 and an Mw of 25,000. Wild-type human TF was purified from brain as described,25 and reconstituted into mixed rabbit brain phospholipid vesicles according to the method of Casar and Konigsberg.26

Blood collection. Blood collection for this project was from volunteer donors who had given written consent, under protocols approved by the Institutional Review Boards of the University of Oklahoma Health Sciences Center and the Oklahoma Medical Research Foundation. Blood was drawn from normal healthy donors directly into sodium citrate anticoagulant using either combination syringe/centrifuge tubes containing 0.5 mL 0.106 mol/L trisodium citrate (Sarstedt, Princeton, NJ), or siliconized glass vacuum collection tubes containing buffered sodium citrate (Becton Dickinson Vacutainer Systems, Rutherford, NJ). A two-syringe collection technique was used: venipuncture was performed using a butterfly catheter (Surflo brand winged infusion set with a siliconized 19-gauge needle; Terumo Medical Corporation, Elkton, MD) through which 5-mL samples of blood were collected sequentially into two separate Monovette syringes or Vacutainer tubes. Unless otherwise indicated, only the second collection tube was used for subsequent analysis. Platelet-poor plasma was prepared from citrated blood by centrifugation at 3,200g for 20 minutes. All handling of blood and plasma was at room temperature using polystyrene or polypropylene centrifuge tubes and pipets; the use of glass vessels or pipets was avoided. Plasma samples were either assayed fresh or were stored frozen at −70°C in plastic containers. Frozen plasma samples were thawed rapidly at 37°C, then held at room temperature until assayed. Pooled human plasma was prepared by mixing together citrated, platelet-poor plasma from 20 normal donors, and storing aliquots at −70°C in polypropylene or polystyrene tubes until used.

Soluble TF reagent. A stock solution of rabbit brain cephalin was prepared in normal saline according to the manufacturer’s instructions (Sigma), resulting in a 10-fold concentrate (10× rabbit brain cephalin [RBC]) relative to the final concentration recommended by the manufacturer for use in the Partial Thromboplastin Time test. The soluble TF reagent was prepared by diluting the 10× RBC concentrate (typically to yield 2× RBC final) with TBS/BSA (100 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.4, 0.02% NaN3, 0.1% bovine serum albumin) containing sTF25-39 at a level sufficient to yield the indicated final concentrations (typically 1 µmol/L sTF25-39 final). Soluble TF reagent was typically stored frozen at −70°C in small aliquots and was never subjected to more than one freeze/thaw cycle.

Phospholipid vesicles were prepared from purified phosphatidylcholine and phosphatidyserine (in a 60:40 molar ratio; PCPS vesicles) or mixed brain phospholipid using either the octylglucoside dialysis method,27 or a modification of the method of Casar and Konigsberg28 in which the tissue factor was omitted.

Soluble TF clotting assay for FVIIa. Unless otherwise indicated, the new FVIIa assay, in either manual or automated mode, was performed using plasma samples that had been diluted 10-fold with FVII-deficient plasma (1 vol test plasma plus 9 vol FVII-deficient plasma).

The soluble TF clotting assay was performed in manual mode as follows: Polystyrene 12 × 75 mm test tubes were prewarmed in a 37°C water bath, along with a solution of 25 mmol/L CaCl2 made up in water. Soluble TF reagent (0.1 mL per tube) was pipetted into each test tube and allowed to warm to 37°C for at least 2 minutes. Room-temperature plasma samples (0.1 mL) were pipetted into the tubes and allowed to warm to 37°C for exactly 30 seconds. Then, 0.1 mL prewarmed CaCl2 solution was pipetted into each tube and rapidly mixed. The clotting time was determined by the manual tilt-tube method, measured from the point of addition of CaCl2. These assays were performed in duplicate.

Automated soluble TF clotting assays were performed using a coagulometer (Automated Coagulation Laboratory model ACL300+ from Instrumentation Laboratories, Lexington, MA) as follows: The instrument was operated in research mode, and sample volumes per assay (plasma, soluble TF reagent, and 25 mmol/L CaCl2 solution) were 50 µL each. Settings were: 64 seconds activation time; 1 second inter-ramp interval; 0 seconds delay; 250 seconds acquisition time; 1,200 rpm rotor speed. Before each performance of the soluble TF clotting assay, the instrument was cleaned with 0.1 N HCl as recommended by the manufacturer to eliminate carry-over of plasma or thromboplastin from previously conducted assays. Data were collected on an IBM microcomputer and analyzed using the Windows Research Software supplied with the coagulometer (Instrumentation Laboratories), with clotting times determined by absorbance threshold (threshold set at 6.5% of baseline). For comparison, some assays were performed using a Fibrometer (Becton Dickinson).
Standard curves for calibration of the soluble TF clotting assay for FVIIa were prepared using citrated, FVII-deficient plasma containing varying final concentrations of either recombinant or plasma-derived human FVIIa (by serial dilution) and performing the clotting assays in either manual or automated mode as described above. The clotting times were plotted versus the concentration of FVIIa on log-log scales, and a polynomial curve was fit to the data by regression analysis using Slide Write Plus version 4.1 (Advanced Graphics Software, Sunnyvale, CA) run on an IBM-compatible microcomputer. The concentrations of FVIIa in unknown plasma samples were calculated from the measured clotting times by performing an iterative solution to the polynomial equation fit to the clotting times of the FVIIa standards (using a computer program written in Turbo Pascal). These values were then multiplied 10-fold to correct for the dilution with FVII-deficient plasma. A new standard curve was prepared for each lot of soluble TF reagent and for each assay configuration. Values for statistical significance (P) were from Student’s t-test.

RESULTS

Dependence of clotting time on FVIIa concentration. sTF2-219, a soluble mutant form of TF consisting only of the extracellular domain, was previously reported to retain cofactor function when tested with purified FVIIa, factor X, and phospholipid vesicles. We recently demonstrated that sTF2-219 has selectively lost the ability to promote the activation of FVII to FVIIa. Therefore, if conversion of FVII to FVIIa during clotting is indeed dependent on this function of wild-type TF, then sTF2-219 should clot plasma based on its pre-existing content of FVIIa, and not on its content of zymogen FVII. In the present study, we have compared the ability of wild-type TF versus sTF2-219 to promote clotting of human plasma to which varying amounts of purified FVIIa had been added (Fig 1, A and B). The clotting time of normal human plasma initiated by wild-type TF was relatively insensitive to the presence of added FVIIa (Fig 1A). In contrast, the clotting times of human plasma initiated by a mixture of sTF2-219 and phospholipid vesicles exhibited a very strong
MEASUREMENT OF ACTIVATED FACTOR VII IN PLASMA

737
dependence on FVIIa levels from approximately 1 ng/mL to 10 µg/mL added FVIIa (Fig 1B). Therefore, sTF<sub>2219</sub> appeared to fit the requirements outlined above for a mutant TF that could be used to develop a new clotting assay specific for plasma FVIIa.

The clotting time curve of normal plasma with sTF<sub>2219</sub> exhibited a plateau at concentrations of added FVIIa that were less than approximately 1 ng/mL (Fig 1B). A likely explanation for this result was that the endogenous FVIIa level of the plasma was on the order of 1 ng/mL. To develop a sTF<sub>2219</sub>-based clotting test capable of measuring absolute FVIIa levels, it was necessary to find a means of calibrating the assay. In theory, this could be accomplished by preparing a standard curve of clotting time versus FVIIa concentration using human plasma that contained little or no baseline FVIIa (ie, FVIIa-deficient plasma). It was reasoned that plasma deficient in FVII should also exhibit correspondingly low levels of plasma FVIIa. When the soluble TF clotting tests were performed with FVII-deficient plasma supplemented with FVIIa, no evidence of plateau in the clotting curve was seen at levels of added FVIIa down to 10 pg/mL (Fig 1C). This indicated that FVII-deficient plasma supplemented with varying concentrations of FVIIa could be used to prepare a standard curve for calibration of the assay. Furthermore, it indicated that the assay would have a remarkable sensitivity range (10 pg/mL to 10 µg/mL FVIIa).

Soluble TF clotting times of normal and hemophilic plasmas (supplemented with FVIIa) exhibited the previously encountered leveling off of the clotting curves at low levels of added FVIIa (Fig 1C). The clotting times of these plasmas determined in the absence of any added FVIIa were used to calculate the endogenous levels of FVIIa by reference to a standard curve prepared from parallel clotting time determinations of FVII-deficient plasma supplemented with purified FVIIa. For the normal and hemophilic plasmas in Fig 1, the endogenous levels of FVIIa were calculated to be 0.89 and 0.12 ng/mL, respectively. These baseline FVIIa values were used to recalculate the actual final concentrations of FVIIa in the plasmas, and the clotting curves were replotted (Fig 1D). After recalibration, the points were found to fit well to the standard curve obtained with FVII-deficient plasma, as would be predicted if the assay were a sensitive and specific measure of plasma FVIIa levels.

The clotting assays described above were conducted in polystyrene test tubes to avoid contact activation. When the assays were performed in unsiliconized borosilicate glass test tubes, shorter clotting times were consistently observed for normal plasma compared with FVII-deficient at all concentrations of added FVIIa tested. Furthermore, data points recalculated to account for "endogenous" FVIIa levels in this case deviated from the curve prepared using FVII-deficient plasma (data not shown). Activation of the contact pathway by glass likely resulted in the conversion of some plasma FVII to FVIIa, which then interfered with the assay. This underscored the necessity of using plastic tubes for the clotting test.

**Manipulation of assay sensitivity.** Clotting times of plasma using sTF<sub>2219</sub> and phospholipid as the procoagulant were found to depend on the concentration of sTF<sub>2219</sub>, with clotting times approaching a minimum at approximately 2 to 5 µmol/L sTF<sub>2219</sub> (Fig 2A). This suggested that sensitivity of the soluble TF clotting assay toward FVIIa could be manipulated simply by changing the sTF<sub>2219</sub> concentration to obtain a convenient range of clotting times with a given expected range of FVIIa levels. Fig 2B shows parallel clotting curves obtained by varying the sTF<sub>2219</sub> concentration. A "high sensitivity" reagent, containing nearly saturating levels of sTF<sub>2219</sub> (1 µmol/L), yielded a range of clotting times convenient for general determination of plasma FVIIa levels in
normal individuals (Fig 2C). Assay sensitivity could also be adjusted by varying the phospholipid concentration, which resulted in a family of parallel clotting curves similar to those of Fig 2C (data not shown). Furthermore, several different types of phospholipid vesicles were tested, including vesicles prepared from purified phosphatidylcholine and phosphatidylserine, from mixed brain phospholipids, and a commercial rabbit brain cephalin preparation marketed for use in the partial thromboplastin time test. All gave equivalent results, indicating that the precise nature of the phospholipid vesicle preparation appeared not to be important.

Lack of contribution of zymogen FVII to clotting times. If zymogen FVII was indeed not converted to FVIIa during clotting induced by sTF$_{2,219}$, it was reasoned that reconstituting FVII-deficient plasma with purified FVII to the levels encountered in normal plasma should have no effect on the observed clotting time. The preparation of FVII used contained undetectable FVIIa by sodium dodecyl sulfate (SDS)-polyacrylamide gel analysis. However, because even highly purified FVII contains traces of FVIIa, it was necessary to measure the level of FVIIa present in our preparation of recombinant FVII. Therefore, trace contamination by FVIIa was assessed as previously described, taking advantage of the fact that sTF$_{2,219}$ enhances the amidolytic activity of FVIIa under conditions where FVII is not converted to FVIIa. First, the amidolytic activity of the recombinant FVII (at 30 to 3,000 nmol/L) was measured in the presence of 4 μmol/L sTF$_{2,219}$. The rate of chromogenic substrate hydrolysis per nanomolar FVII was then compared with a standard curve of the rate of substrate hydrolysis versus FVIIa concentration that had been prepared by performing the identical procedure, except that varying concentrations of purified FVIIa were used, as previously described. From this analysis, it was calculated that the recombinant FVII preparation contained 0.475 ± 0.033 mole percent FVIIa. Recombinant FVII was then added to congenital FVII-deficient plasma to a final concentration of 500 ng/mL, and clotting times were determined using soluble TF reagents composed of 1 μmol/L sTF$_{2,219}$ and either 2× RBC or 100 μmol/L PCPS vesicles. The clotting times (36.7 and 38.7 seconds, respectively) determined using an automated coagulometer were then converted to FVIIa concentrations by comparison with standard curves of soluble TF clotting times that had been prepared by adding known concentrations of FVIIa to the FVII-deficient plasmas (resulting in standard curves similar to those of Fig 2C). The clotting times for the FVII-supplemented, FVII-deficient plasma obtained with the two soluble TF reagents corresponded to FVIIa concentrations of 2.5 and 2.2 ng/mL, respectively. Expressed as percent of added FVIIa, these values corresponded to 0.50% and 0.44%, respectively, which agree well with, and more importantly are not significantly higher than, the value of 0.475% FVIIa determined by the amidolytic assay. This provides independent evidence that significant amounts of zymogen FVII were not converted to FVIIa when clotting was promoted by the soluble TF reagent.

Configuration of the soluble TF clotting assay for FVIIa, stability of FVIIa in citrated plasma, and cold activation of FVII. For the remainder of this study (unless otherwise noted), the soluble TF clotting assay for measuring FVIIa levels was performed using a soluble TF reagent consisting of 1 μmol/L sTF$_{2,219}$ and 2× RBC. Test plasma samples were routinely diluted 10-fold with FVII-deficient plasma. Although the assay can be performed on undiluted plasma samples, this was not routinely used because of concern that individual variation in plasma components other than FVIIa (such as levels of factor X, prothrombin, fibrinogen, various inhibitors, etc) might have an influence on the observed clotting times. Therefore, diluting the test plasma samples with FVII-deficient plasma would mean that 90% of the levels of all clotting factors will be contributed by the FVII-deficient plasma; therefore, this should dimish the effect of variation in levels of factors other than FVIIa. Standard curves were generated using FVII-deficient plasma supplemented with varying concentrations of FVIIa (typically 0.03, 0.3, and 30 ng/mL final concentration). Both immunodepleted and congenital FVII-deficient plasma were found to give equivalent results, as did the use of either plasma-derived or recombinant FVIIa for generation of standard curves. In all cases, the same lot of FVII-deficient plasma used for dilution of test plasmas was also used for generating the corresponding FVIIa standard curve.

It was of interest to determine the stability of plasma FVIIa in regard to the FVIIa assay. In a variety of different experiments, plasma samples were assayed immediately following collection, after storage at −70°C, or after incubation at ambient temperature for 2 hours in plastic test tubes. The results agreed within 10% of the initial values (data not shown).

The FVII activity level of plasma samples has been reported to increase during storage at cold temperatures (above freezing but significantly below room temperature), in a process known as cold activation. Cold activation of plasma is thought to reflect the slow conversion of plasma FVII to FVIIa mediated by the contact pathway of blood clotting. This process was examined with the new soluble TF assay, using citrated human plasma from three volunteers that had either been assayed immediately following collection, or incubated 5 hours at room temperature, 37°C, or on ice (Table 1). The effect of incubation of these plasmas in polystyrene, untreated borosilicate glass, or siliconized glass containers was also assessed. Incubation in plastic or siliconized glass tubes at any of the three temperatures in general did not result in significant increases in measured plasma FVIIa levels, with the exception of donor no. 1, whose measured FVIIa levels increased from 1.2 to 2.1 ng/mL following a 5-hour incubation on ice in siliconized glass. However, when these plasma samples were incubated in untreated glass tubes marked elevations in measured FVIIa levels were observed for all three temperatures. This effect was most striking in samples that had been incubated in untreated glass containers on ice, resulting in up to 12-fold increase in FVIIa concentration.

Effect of heparin on determination of FVIIa levels. Because it may be desirable to determine FVIIa levels in individuals who have received heparin treatment, it was of interest to examine the potential effect of heparin on measured FVIIa levels. Accordingly, heparin was added at varying concentrations to a citrated plasma sample, and the soluble TF clotting assay was performed. As can be seen from Table
MEASUREMENT OF ACTIVATED FACTOR VII IN PLASMA

Table 1. FVIIa Levels in Plasma After Incubation in Glass or Plastic Containers

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>Donor 1</th>
<th>Donor 2</th>
<th>Donor 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Polystyrene</td>
<td>Siliconized Glass</td>
<td>Untreated Glass</td>
</tr>
<tr>
<td>Ice bath</td>
<td>3.4</td>
<td>3.3</td>
<td>1.0</td>
</tr>
<tr>
<td>37°C</td>
<td>1.0</td>
<td>2.6</td>
<td>3.7</td>
</tr>
<tr>
<td>Ambient</td>
<td>0.8</td>
<td>3.7</td>
<td>3.6</td>
</tr>
<tr>
<td>37°C</td>
<td>1.7</td>
<td>4.0</td>
<td>3.6</td>
</tr>
<tr>
<td>Ambienet</td>
<td>4.1</td>
<td>4.0</td>
<td>4.1</td>
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<tr>
<td>37°C</td>
<td>3.0</td>
<td>4.0</td>
<td>4.0</td>
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</table>

FVIIa levels (in ng/mL) were measured with the soluble TF assay using citrated plasma samples that were assayed immediately after collection (initial), or following incubation for 5 hours at the specified temperatures (ambient temperature was approximately 22°C). Siliconized Glass, test tubes (both had been rinsed with deionized water and air-dried before use). Untreated Glass, borosilicate glass citrated plasma samples that were assayed immediately after collection. FVIIa, the assay was performed five times per day on 10 plasma samples obtained from three donors on three sequential days (Table 3). One donor (no. 1) had low but relatively constant levels of measured FVIIa on all sequential days (Table 3). One donor (no. 1) had low but relatively constant levels of measured FVIIa on all sequential days (Table 3). One donor (no. 1) had low but relatively constant levels of measured FVIIa on all sequential days (Table 3). One donor (no. 1) had low but relatively constant levels of measured FVIIa on all sequential days (Table 3). One donor (no. 1) had low but relatively constant levels of measured FVIIa on all sequential days (Table 3). One donor (no. 1) had low but relatively constant levels of measured FVIIa on all sequential days (Table 3). One donor (no. 1) had low but relatively constant levels of measured FVIIa on all sequential days (Table 3). One donor (no. 1) had low but relatively constant levels of measured FVIIa on all sequential days (Table 3).

Table 2. Effect of Added Heparin on Measurement of Plasma FVIIa

<table>
<thead>
<tr>
<th>Heparin, U/mL</th>
<th>FVIIa, ng/mL</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>4.2</td>
</tr>
<tr>
<td>0.1</td>
<td>3.9</td>
</tr>
<tr>
<td>0.2</td>
<td>3.9</td>
</tr>
<tr>
<td>0.4</td>
<td>4.0</td>
</tr>
<tr>
<td>0.6</td>
<td>4.0</td>
</tr>
<tr>
<td>1.0</td>
<td>4.0</td>
</tr>
<tr>
<td>5.0</td>
<td>No clot</td>
</tr>
</tbody>
</table>

Immediately before measuring FVIIa levels, heparin was added to aliquots of a citrated human plasma to the indicated final concentrations.

If this hypothesis were correct, it would be expected that variation in FVIIa levels would be random, and not a characteristic of a given individual. To test the source of individual variation in plasma FVIIa levels, samples from the donors in Table 3 were obtained by phlebotomy simultaneously from both the left and right arms (day 3). The measured FVIIa levels of the duplicate samples for donor nos. 1 and 2 were identical, while the levels measured in the samples from donor no. 3 differed by 7.4% from each other. In additional experiments, FVIIa levels were determined in several plasma samples taken from the first and second syringes obtained from the same needle puncture. Differences between FVIIa levels in the first and second syringes was always less than 10% (data not shown). Therefore, differences in FVIIa levels appear to be a characteristic of the individual donors, and are highly unlikely to be caused by random FVIIa generation during phlebotomy.

Variation in FVIIa levels among individuals in a study population. In an initial assessment of individual variation of plasma FVIIa levels, a population study was undertaken in which FVIIa concentrations were measured in plasma samples obtained from 200 healthy donors. Statistics on the age distribution, smoking status, and use of oral contraceptives of individuals in the study population is given in Table 4. Excluding pregnant women, the overall mean plasma FVIIa concentration for the population (188 donors) was 3.58 ng/mL with a standard deviation of 1.44 ng/mL. The range of FVIIa concentrations was very large, varying nearly 17-fold from a low of 0.5 ng/mL to a high of 8.4 ng/mL. Expressed as percentage of the mean, this corresponds to a range of FVIIa levels of 14.0% to 235% of the mean value. Exclusion of smokers and users of oral contraceptives from analysis left a population of 87 individuals with a mean FVIIa concentration of 3.73 ng/mL and a standard deviation of 1.44 ng/mL.

Table 3. Day-to-Day Variation in FVIIa Levels in Three Individuals

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
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<tbody>
<tr>
<td>Donor 1</td>
<td>1.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Donor 2</td>
<td>4.3</td>
<td>4.7</td>
</tr>
<tr>
<td>Donor 3</td>
<td>3.9</td>
<td>3.1</td>
</tr>
</tbody>
</table>

Plasma FVIIa levels (in ng/mL) determined on 3 successive days using the soluble TF assay. On day 3, the two values for FVIIa are for samples drawn simultaneously from both the right and left arms.

2, addition of heparin to the test plasma at levels up to 1 U/mL had no significant effect on the observed FVIIa levels. Thus, although high levels of heparin (5 U/mL) interfered with the assay, therapeutic levels of heparin (0.1 to 0.5 U/mL in plasma) should not interfere with determining FVIIa levels using the new assay. The relative insensitivity of the assay to heparin is probably caused, in part, by the 10-fold dilution of the test plasma with FVII-deficient plasma. Individual variation in measured FVIIa levels. To establish the precision of the new soluble TF clotting assay for FVIIa, the assay was performed five times per day on 10 successive days using aliquots of citrated plasma derived from a single collection from a single donor (stored frozen at −70°C). The overall mean plasma FVIIa for this donor was found to be 4.14 ng/mL, with coefficients of variation for within-day and between-day variability of 2.2% and 8.1%, respectively.

Day-to-day variation in FVIIa levels were determined on plasma samples obtained from three donors on three sequential days (Table 3). One donor (no. 1) had low but relatively constant levels of measured FVIIa on all 3 days, whereas another donor (no. 2) had higher levels that increased somewhat (by 38%) on the third day. The third donor had levels that approximately doubled on day 3. This donor had a history of peptic ulcer, and experienced an episode of acute gastritis on day 3; however, the significance of this condition to the increase in measured FVIIa levels is unknown.

It was important to address the possibility that plasma FVIIa could be the result of a phlebotomy artifact. It was conceivable, for example, that artifactual generation of FVIIa occurred during blood drawing (as a consequence of transient activation of the clotting cascade at the site of insertion of the needle), and that this accounted for the measured FVIIa.
mL. The observed range of FVIIa concentrations in this subpopulation was 0.5 ng/mL to 6.9 ng/mL, corresponding to a range of 13.4% to 186% relative to the mean value.

Variations in FVIIa levels are depicted in Fig 3, sorted according to sex, smoking status, and use of oral contraceptives. In this study population (excluding pregnant women), no significant difference between FVIIa levels of males versus females was observed. Males had a mean FVIIa level of 3.8 ± 1.37 ng/mL, whereas females had a mean of 3.64 ± 1.51 ng/mL. In addition, no significant difference was observed when the study population was divided according to smoking status (smokers, mean plasma FVIIa of 3.55 ± 1.41 ng/mL; nonsmokers, 3.60 ± 1.46 ng/mL; P > .5) or use of oral contraceptives (oral contraceptive users, mean plasma FVIIa of 3.31 ± 1.42 ng/mL; not oral contraceptive users, 3.50 ± 1.52 ng/mL; P > .25). Indeed, when FVIIa levels were compared among all the respective sub-groups, the only difference that approached statistical significance was between female nonsmokers who did or did not use oral contraceptives (oral contraceptive users, mean plasma FVIIa of 3.03 ± 1.41 ng/mL; not oral contraceptive users, 3.64 ± 1.51 ng/mL; P = .06). However, because the mean ages differed substantially between the two sub-groups (nonsmoking women who did or did not use oral contraceptives), it is possible that the difference in mean FVIIa levels between the two could reflect the difference in mean age rather than use of oral contraceptives. As can be seen from Fig 4, there was a significant correlation between FVIIa levels in both males and females with increasing age (r = .40, P = .009, and r = .34, P = .03, respectively).

Correlation between FVIIa levels and other parameters of the clotting system. The FVII:C assay is thought to measure an aggregate of both FVII and FVIIa in plasma. Therefore, it was of interest to compare FVII:C levels to FVIIa levels (Fig 5). A positive correlation between FVII:C and FVIIa levels was observed both in males and females (r = .82, P < 10⁻⁶, and r = .84, P < 10⁻⁶, respectively). However, for a given FVII:C level, FVIIa varied by as much as 2.5-fold, and only 50% of the points fell within the 95% confidence interval of the correlation (Fig 5).

As depicted in Fig 5, no significant correlation was observed between plasma fibrinogen and FVIIa levels in males or females.
MEASUREMENT OF ACTIVATED FACTOR VII IN PLASMA

Fig 5. Relationship between plasma FVIIa levels and FVII:C values for normal males and females (nonsmokers, not using oral contraceptives). The study population is described in Table 4, and FVIIa levels were determined as described in the legend to Fig 3. Regression line is solid for males (●), dashed for females (○).

Fig 6. Lack of correlation between plasma fibrinogen and FVIIa levels in normal males and females (nonsmokers, not using oral contraceptives). The study population is described in Table 4, and FVIIa levels were determined as described in the legend to Fig 3. Males (●), females (○).

Fig 7. Relationship between prothrombin time and plasma FVIIa levels in normal males and females (nonsmokers, not using oral contraceptives). The study population is described in Table 4, and FVIIa levels were determined as described in the legend to Fig 3. Males (●), females (○).

Fig 8. FVIIa levels in nonsmoking, pregnant women compared with age-matched, nonsmoking women (not using oral contraceptives). Plasma samples were obtained during uncomplicated pregnancies from 12 healthy women (P) ranging in age from 18 to 38 years old, while plasma FVIIa values for age-matched control women (NP) were taken from the study population described in Table 4. Samples were obtained during the first (△), second (●), or third (○) trimester of pregnancy. All FVIIa levels were determined as described in the legend to Fig 3; values for the pregnant women were determined at the same time, using the same batch of reagents, as the nonpregnant women.

FVIIa levels and pregnancy. FVIIa levels were determined in 12 healthy pregnant women, none of whom smoked. Figure 8 depicts a comparison of FVIIa levels between these pregnant women and age-matched, nonpregnant women (nonsmokers who were not taking oral contraceptives). Pregnant women had mean FVIIa levels of 5.35 ± 2.06 ng/mL. This was significantly higher than nonpregnant control women, who had mean FVIIa levels of 3.13 ± 0.74 ng/mL (P = .002).

Effect of oral anticoagulant therapy on FVIIa levels. The effect of oral anticoagulant therapy on plasma FVIIa was examined in daily plasma samples obtained from individuals who had been diagnosed with acute deep vein thromboses, as they were placed on 5 mg/d oral warfarin. As can be seen from Fig 9, FVIIa levels in three representative patients decreased rapidly to their lowest levels within 5 to 7 days after initiation of warfarin therapy. Interestingly, FVIIa levels decreased to a nadir before the prothrombin times reached their...
three panels represent three individuals with acute, venographically documented, deep vein thrombosis placed on 5 mg/d oral warfarin immediately before first dose of warfarin. Daily plasma FVIIa levels (E) were determined as described in the legend to Fig 3, and prothrombin times (C) are given for comparison. The lower limit of sensitivity for FVIIa in this assay was 0.3 ng/mL; triangles (a) correspond to <0.3 ng/mL FVIIa.

**DISCUSSION**

Although the existence of low levels of FVIIa in plasma was postulated, plasma FVIIa has not been measurable directly because of limitations in existing assay techniques. The main technical problem has been that TF strongly promotes the conversion of FVII to FVIIa, meaning that both FVII and FVIIa contribute to the FVII:C assay. However, we have recently demonstrated that a soluble mutant form of TF has selectively lost the ability to promote activation of plasma FVII. The effective range of the assay is very large, extending from 10 pg/mL to as much as 10 μg/mL FVIIa. Control experiments established that artifactual generation of FVIIa during phlebotomy, if it took place, did not contribute significantly to the measured FVIIa levels.

Citrated plasma has been known to undergo "cold activation" if stored at temperatures just above freezing, especially in glass containers. Cold activation is reflected in an increase in FVII:C levels, and has been hypothesized to be a consequence of activation of plasma FVII via the contact pathway of blood clotting. In this study, we have measured directly the increase in FVIIa levels during storage of plasma samples on ice in glass containers. Therefore, the new FVIIa assay should be useful in studying the process of cold activation in greater detail.

Elevated plasma FVII:C levels, along with elevated plasma fibrinogen, have been found to be significant predictors of risk of ischemic heart disease and cardiovascular death in epidemiologic studies. However, FVII:C assays have been performed differently in different laboratories, and the degree of sensitivity of the FVII:C assay to FVII versus FVIIa is thought to vary depending on assay configuration. Indeed, it is controversial whether or not the important factor in elevated FVII:C levels is elevation in plasma FVIIa per se, or elevation in total FVII mass. Thus, it would clearly be desirable to evaluate FVII and FVIIa levels separately. Total plasma FVII can already be measured with existing technology, either by enzyme-linked immunosorbent assay (ELISA) methods, or by activity assays in which the bulk of FVII is converted to FVIIa, such as FVII amidolytic assays (discussed by Hoffman et al). The ratio of FVII:C to total FVII mass has been used to try to obtain an indication of the degree of activation of plasma FVII. However, this method relies on assumptions concerning what FVII:C is measuring, is subject to variability in sensitivity of FVII:C to FVIIa, and does not yield an absolute measure of FVIIa levels. The novel assay described in this study now provides the means to rapidly and directly determine plasma FVIIa levels, and therefore should be useful in answering the question of the role of FVIIa per se as a predictor of risk in disease.

Although an extensive epidemiologic study of the association of disease risk with FVIIa levels is clearly outside the scope of the present communication, we undertook an initial population study to determine the degree of individual variation in FVIIa levels, and to examine potential correlations between FVIIa levels and such parameters as age, sex, smoking, use of oral contraceptives, and pregnancy. This study confirms the earlier prediction that plasma might normally contain trace levels of pre-existing FVIIa, and furthermore provides previously unavailable quantitation of plasma FVIIa levels. Plasma FVIIa values were found to vary substantially between individuals—among 188 volunteers' FVIIa levels varied nearly 17-fold, from a low of 0.5 ng/mL to a high of 8.4 ng/mL, with a mean of 3.58 ± 1.44 ng/mL. Taking the mean concentration of total plasma FVII + FVIIa in humans to be 470 ng/mL, the mean plasma level of FVIIa found in this study would represent 0.76% of the total FVII mass. No significant differences in FVIIa levels were observed between men and women, nor was there a clear correlation between FVIIa levels and smoking status or use of oral contraceptives in this population. A significant correlation between FVIIa levels and increasing age was found, although substantial
variation between individuals was observed even within age groups.

Based on a preliminary presentation of our new assay in abstract form,21 Wildgoose et al22 used the soluble TF assay to measure FVIIa levels in a normal and hemophilic population, reporting a mean FVIIa level in normal individuals (4.34 ± 1.57 ng/mL) similar to that found in the present study. Wildgoose et al22 also found FVIIa levels to be slightly decreased in severe factor VIII-deficient patients, and substantively decreased in severe factor IX-deficient patients. However, their study did not report the age distribution of the study populations; given the age dependence of FVIIa levels found in the present study, it would be important to ensure that hemophilic patients are compared with an age-matched control group.

Potential correlations between plasma FVIIa levels and other parameters of the clotting system were investigated in the present study. FVIIa levels exhibited no significant correlation with plasma fibrinogen. This is interesting in light of epidemiologic studies associating both elevated plasma fibrinogen and FVII:C levels with increased risk of heart disease, and the fact that fibrinogen levels have been observed to be increased in smokers versus nonsmokers (reviewed by Hultin16 and Ernst23). Thus, if plasma FVIIa is associated with risk of heart disease, it would constitute an independent risk factor relative to plasma fibrinogen levels, smoking, or use of oral contraceptives. This opens the possibility that combined risk estimates of fibrinogen levels (or smoking, contraceptive use, etc) and FVIIa levels may constitute a more powerful means of determining risk of thrombosis.

Plasma FVIIa levels exhibited a relatively weak but positive correlation with the results of the FVII:C test. This was expected, given that FVII:C is thought to measure both FVII and FVIIa in plasma. FVIIa levels were found to be negatively correlated with prothrombin times (as were FVII:C levels; data not shown). If plasma FVIIa serves a priming function in TF-mediated triggering of the clotting cascade, prothrombin times should be shortened in the presence of increasing FVIIa. Indeed, previous studies have shown that adding purified FVIIa to plasma results in shortening of the prothrombin time.34 As with the relationship between FVIIa levels and FVII:C, however, the correlation was not perfect. This is also not surprising, considering that the prothrombin time is sensitive to variation in the levels of several different clotting factors.

FVIIa levels decreased rapidly and dramatically in patients with deep vein thrombosis as they underwent oral anticoagulation therapy. If FVIIa proves to be a marker of risk of additional thromboses, measuring FVIIa levels might be an effective means of monitoring warfarin dosage in such patients.

Pregnancy has previously been reported to be associated with substantial increases in FVII:C levels,30,35,36 and the question has been raised as to a possible association between high plasma FVII activity and fetal growth retardation.35 In the present study, we have found that FVIIa levels are significantly increased during normal pregnancy.

The new assay will now permit direct analysis of the role of FVIIa as a risk predictor for thrombosis. In particular, it will be important in future studies to evaluate the role of FVIIa per se for conditions associated with elevated FVII:C levels, including risk of ischemic heart disease and fetal growth retardation. In addition, it will be important to determine the diurnal variation in FVIIa and the effect of serum triglyceride levels16,17,19,37,38 on the determination of FVIIa using the new assay. Furthermore, the new assay could be used to measure the effective plasma dose of FVIIa in individuals receiving recombinant FVIIa therapy for bleeding disorders.22 It will also be of interest to compare FVIIa levels in a variety of clinical and experimental settings to measurements of the degree of in vivo activation of other proteases of the clotting cascade, such as assays that measure plasma levels of protease-inhibitor complexes, F1+2, or activation peptides released from various other clotting factors.30,31

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Quantitation of activated factor VII levels in plasma using a tissue factor mutant selectively deficient in promoting factor VII activation

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