Altered Factor VII Activity in Hemophilia

By Barbara C. Miller, Mae B. Hultin, and Jolyon Jesty

Factor VII levels have been studied in hemophilia A and B plasmas and normal controls in a controlled, prospective study. Three assay methods were used: (1) a standard clotting assay (FVIIc-A); (2) a modified clotting assay (FVIIc-B) (Seligsohn et al, Blood 52:978–988, 1978); and (3) a coupled amidolytic assay. By the FVIIc-B assay, the hemophilic plasmas were significantly lower than in the normal group (68.2 ± 3.3% [SE] and 83.5 ± 3.8%, respectively; P < .01). The amidolytic assay, however, which measures total factor VII regardless of its activity state (factor VII or VIIa), was higher in the patient group than in the control group (126.9 ± 9.6% and 99.4 ± 5.7%, respectively; P < .01). Control experiments showed that the differences in FVIIc-B activity were not caused by artifactual activation of factor VII in vivo in the control group. The mean FVIIc-A assay of hemophilic plasmas (126.3 ± 6.5%) agreed closely with the amidolytic assay, suggesting that the FVIIc-A method is also insensitive to the factor VII activity state. These data support the hypothesis that the FVIIc-B assay is more sensitive to the presence of factor VIIa. The increased sensitivity of the FVIIc-B assay to factor VII activation was confirmed by comparison of the two clotting assays on plasma subjected to activation in glass at 4 °C. The results of this study indicate that factor VII in hemophilic plasma is less activated than in normal plasma. Whether this contributes to the bleeding diathesis of hemophilia is unknown. However, it does provide evidence for the idea that factor VII in vivo is normally subject to some degree of activation by an enzyme (or enzymes) generated by a turnover of the intrinsic pathway.

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Factor VII is the only plasma-clotting factor whose effect is exerted primarily in the extrinsic pathway of coagulation. To activate its principle substrates—factor IX or X—at maximum rate in the presence of tissue factor, factor VII must be activated to the two-chain enzyme, factor VIIa. In 1978, Seligsohn et al described a method that detected factor VIIa in plasma by comparison of a clotting assay—which they showed was sensitive to factor VIIa—with a coupled amidolytic assay, which was sensitive only to total factor VII concentration. The clotting assay Seligsohn et al used, which in this paper we arbitrarily designate FVIIc-B, differs in several respects from a more widely used clotting assay for factor VII, which we designated FVIIc-A. In normal plasma, the ratio of factor VII clotting activity (FVIIc-B) to factor VII amidolytic activity (FVIIam) was approximately 1, whereas in plasma activated to generate factor VIIa, the ratio of FVIIc to FVIIam was increased. Thus, Seligsohn et al concluded that the ratio of FVIIc-B to FVIIam is an indication of the factor VII activity state. In a subsequent paper, they reported a mean FVIIc-B of 73 ± 6% (SE) and a FVIIam of 128 ± 9% in nine hemophilia B patients (ratio, 0.57), without commenting on the significance of these apparent deviations from normal. These results suggested to us that the absence of factor IX or VIII may lead to a decreased activity state of factor VII. We chose to test this by assaying factor VIIc and factor VIIam in a group of hemophilic patients and in a control group of normal males.

Considerable data support the presence of interrelationships between the intrinsic and extrinsic pathways, and factor VIIa has been shown in vitro to activate both factors IX and X in the presence of tissue factor. It has also been shown that single-chain bovine factor VIII possesses a low level of esterolytic activity, and it is possible that in the presence of tissue factor it activates factor IXa and possibly factor X. Factor VII itself can be activated by a number of clotting enzymes: the most potent on a molar basis in vitro is factor Xa, followed by factor IXa and the poor activators factor XIIa and thrombin. Because there is no earlier step in the extrinsic pathway, such baseline levels of factor VII activity—from either the zymogen or trace levels of VIIa—are essential for its initiation. Changes in FVIIc activity in plasma samples such as we describe may reflect an altered concentration of factor VII or VIIa or both. The marked susceptibility of factor VII to activation, and the absence of a significant plasma inhibitor of human factor VIIa, make it quite feasible that low levels of factor VIIa circulate in plasma. We hypothesized that hemophilic subjects have a lower level of plasma factor VIIa than do normal subjects.

MATERIALS AND METHODS

Reagents

Human and bovine thromboplastin were prepared as saline extracts of acetone powder of the appropriate brain by the method of

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Quick. The human brain extract was then diluted with 0.1 mol/L NaCl-0.05 mol/L Tris/HCl pH 7.45 (TBS) to give a prothrombin time with normal human plasma of 17 seconds. Factor VII-deficient plasma and pooled normal plasma (PNP) were purchased from George King Biomedical, Overland Park, Kan. This PNP was obtained from 11 men and 11 women, healthy subjects on no medication. Simplastin was obtained from General Diagnostics, Jessup, Md. For the FVIIam assay, the Simplastin was reconstituted with water, sonicated, and centrifuged at room temperature for eight minutes at 2,000 g. Bovine serum albumin (BSA), Tris, and disodium EDTA were obtained from Sigma, St Louis. Purified human factor IX was prepared by the method of Morrison and Jesty, and human factor VIII-vWF was prepared as previously reported. Bovine factor X was prepared by the method of Jesty and Nemerson. Oxalated bovine plasma was obtained from Pel-Freez, Rogers, Ark, and was adsorbed with 10% BaSO₄ (Fisher, Springfield, NJ) at room temperature for 30 minutes. The BaSO₄ was removed by centrifugation at room temperature for 15 minutes at 2,500 g. The factor Xa chromogenic substrate Ile-Glu-Gly-Arg-p-nitroanilide (S-2222) was obtained from Helena Labs, Beaumont, Tex.

Subjects

The protocol for blood drawing was approved by the Committee on Research in Human Subjects at SUNY, Stony Brook, and written informed consent was obtained from each volunteer donor. A prospective controlled study of eight hemophilic subjects, randomly recruited, and ten controls was initiated and completed within three months. The control group consisted of ten healthy men, all volunteers on no medication (mean age, 27.7 years). In the initial experimental group there were three hemophilia A patients and five hemophilia B patients (mean age, 26.8 years). Inhibitors were present in two hemophilia A patients and in one hemophilia B patient. Since the initial controlled study, six more hemophilic subjects have been studied, and four additional normal subjects have also been studied. No hemophilic subject had received factor infusions within 72 hours of blood sampling, and all were clinically stable, without evidence of bleeding.

Plasma Preparations

Venous blood was drawn into a plastic syringe, added to 1:10 volume of 3.8% sodium citrate, and centrifuged at 4 °C at 3,000 g for 15 minutes. The plasma was removed and immediately frozen at −70 °C in aliquots in plastic vials. In addition, blood was drawn from four normal male volunteers on no medication by a double-syringe technique and similarly citrated. The PNP used as the daily reference in this study was obtained from six men and five women, healthy volunteers on no medication. It was standardized by duplicate factor VII assays against the PNP (22 subjects) from George King Biomedical, which was arbitrarily assigned a value of 100% factor VII. With each set of assays (whether clotting or amidolytic), one coded dilution (60%, 80%, or undiluted) of a single normal donor's plasma was also assayed to serve as a daily internal control.

Clotting Assays

Two clotting assays were performed, both with a Fibrometer (Becton Dickinson, Cockeysville, Md) at 37 °C. Standard factor VII clotting assay (VIIc-A). One hundred microliters of test sample was warmed with 100 μL of factor VII-deficient plasma for five minutes at 37 °C in a plastic Fibrometer cup. The assay was started by the addition of 200 μL of Simplastin at 37 °C. Standard and test samples were appropriately diluted in TBS. Factor VII clotting assay of Seligsohn et al (VIIc-B). The method of Seligsohn et al was slightly modified to accommodate the total reaction volume required by the Fibrometer. Fifty microliters of factor VII-deficient plasma was warmed with 50 μL of adsorbed oxalated bovine plasma and 100 μL of human thromboplastin for three minutes at 37 °C. Fifty microliters of test material was then added, followed after 30 seconds by the addition of 50 μL of 60 mmol/L calcium chloride at 37 °C to start the assay. Samples were diluted in a solution of 0.15 mol/L NaCl-0.05 mol/L Tris/HCl, pH 7.5. The final volume was 300 μL, compared to a final volume of 350 μL in the original method. It is relevant to note that this assay is slightly deficient in factor IX relative to the VIIc-A assay, the ratio of factor IX-containing plasma to total assay volume being 1/6 in the VIIc-B assay v 1/4 in the VIIc-A assay (see Results).

Amidolytic Assay (FVIIam)

The coupled amidolytic assay we have devised was derived from the method of Seligsohn et al. Both stages were performed at 37 °C. In the initial step, 100 μL diluted sample was warmed with 100 μL bovine factor X, 50 μg/mL, for three minutes. The first stage was started by the addition of 200 μL Simplastin. After exactly three minutes, the reaction was stopped by the addition of 100 μL of 0.15 mol/L EDTA/NaOH, pH 7.5. In the second step, a 200-μL subsample of the first incubation was added to a cuvette containing 100 μL of 1 mmol/L S-2222 and 100 μL of TBS-0.1% BSA. The rate of increase of absorbance, measured at 405 nm, obtained by linear regression of absorbance vs time, was plotted against the concentration of plasma standard. The reference dilutions of PNP were 0.008, 0.006, 0.004, and 0.002 in TBS, and TBS alone. Test samples were diluted to 0.006, 0.004, and 0.002. The mean FVIIam for ten normal subjects (seven women and three men) was 102.2 ± 1.2%, compared with 103.5 ± 1.3% for the VIIc-B assay. These ten normal subjects were assayed before the prospective controlled study was started and were not the same subjects as those recruited for the controlled trial.

Prospective Controlled Study

For each assay (FVIIc-A, FVIIc-B, and FVIIam), a single lot or batch of each reagent was used for all of the assays of the initial eight hemophilic and ten normal control subjects, and samples were batched so that each run included normal and hemophilic samples. The additional six hemophilic subjects and four normal subjects were assayed in some cases with different lots of reagents and were not assayed as paired batches in every case.

Statistical Analysis

Significant values were calculated by the paired Student’s t test for two different assays on the same subject group, and by Student’s t test for independent samples for the same assay on two independent groups (patient and normals). Variation of the means is reported as the SE; P < .05 was considered significant.

RESULTS

The results of the three factor VII assays in the prospective controlled study are shown in Fig 1. The mean values for the ten normal subjects were 99.4 ± 5.7% (SE) for the FVIIam assay, 98.4 ± 5.3% for the FVIIc-A assay, and 83.5 ± 3.8% for the FVIIc-B assay. Although none of these values was significantly different from another, the somewhat lower value for the FVIIc-B assay suggested a trend. The mean values in the eight hemophilic patients for the FVIIam, FVIIc-A, and FVIIc-B were 126.9 ± 9.6%, 126.3 ±
Factor VII activities of normal and hemophilic plasmas. FVIIc-A, FVIIc-B, and FVIIam were assayed in a group of ten normal men (N) and eight subjects with hemophilia (H). Assay methods are described in Materials and Methods. Error bars show SEM.

6.5%, and 68.2 ± 3.3%, respectively. In each case, the mean value for the hemophilic patients was significantly different from the corresponding mean value for the normal controls (P < .01). In addition, while the FVIIc-A and FVIIam assays in the hemophilic group agreed closely, the FVIIc-B assay was significantly different from both of them (P < .001). Since this controlled prospective study, we have assayed six additional hemophilic subjects (three who were factor IX-deficient and three who were factor VIII-deficient), and we have also assayed four additional normal subjects. The results for this larger group of subjects (14 hemophilic subjects and 14 normal subjects) were very similar to the initial study group (Table 1) and yielded the same statistical significance. The mean FVIIc-B level for the normal group (92.0%) was closer to the FVIIam level than for the smaller group of normals in the prospective trial, suggesting that this difference resulted from small sample size. Our interpretation of these data was that subjects with hemophilia had a significantly decreased factor VII activity state, measured by the FVIIc-B-FVIIam ratio, and possibly an increased total factor VII concentration. However, another possible explanation for the data was that activation of factor VII occurred after drawing the blood in the normal, but not the hemophilic, plasmas. To evaluate this possibility, blood from four normal men was drawn by a double-syringe method and tested by the FVIIc-B assay. The mean FVIIc-B activity of these plasmas was 81.5% as compared to 83.5% for the ten normals drawn by the single-syringe method, showing that the method of drawing blood did not affect the results.

It is also possible that the FVIIc-B assay on hemophilic plasma was artifactually decreased because of the relative lack of factor IX in this method, compared with the FVIIc-A assay (Materials and Methods). To test this, we added back purified human factor IX, to achieve a level of 1 U/mL, to the plasma of one hemophilia A subject and two hemophilia B subjects and found no increase in the FVIIc-B assay. It is therefore clear that the low level of factor IX in the assay was not the cause of the low factor VIIc-B levels in the subjects with hemophilia.

These results suggested that the FVIIc-B assay may be measuring a real difference in the hemophilic plasma that was not detectable by the FVIIc-A assay. To establish further that the FVIIc-B assay is more sensitive to the activation of factor VII, we performed two experiments involving the generation of factor Vlla by activation of factor VII in plasma in the cold. On two separate occasions, freshly drawn plasma from two normal women taking oral contraceptives was allowed to activate in glass tubes at 4 °C for 24 hours. Factor VII activity was measured by all three assay methods before and after activation in the duplicate experiments (Table 2). As we expected, the amidolytic assay showed no change with activation, but both clotting assays showed an increased factor Vlla in both plasma samples upon activation. Significantly, though,

<table>
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<th>Table 1. Factor VII Assays in Hemophilic and Normal Subjects</th>
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<td><strong>Hemophilic Subjects</strong></td>
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<td>-------------------------</td>
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<tr>
<td>Prospective, controlled study</td>
</tr>
<tr>
<td>FVIIc-A</td>
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<tr>
<td>P &lt; .001</td>
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<tr>
<td>FVIIc-B</td>
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<tr>
<td>P &lt; .001</td>
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<td>FVIIam</td>
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<td>All subjects studied</td>
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<tr>
<td>FVIIc-A</td>
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<td>P &lt; .001</td>
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<td>FVIIc-B</td>
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<td>FVIIam</td>
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the FVIIc-B assay was two to four times more sensitive to factor VII activation than the FVIIc-A assay.

In an attempt to elucidate the critical difference between the FVIIc-A and FVII-B methods, various modifications of the FVIIc-B method were evaluated with hemophilic plasmas. The order of addition of reagents, the sources of thromboplastin (rabbit or human) and factor VII-deficient plasma (three donors tested), and the incubation times were each systematically altered to make the FVIIc-B method more closely resemble the FVIIc-A method. Changing the source of thromboplastin or factor VII-deficient plasma had no effect. When the FVIIc-B assay was performed in the same order of reagent addition and with the same incubation times as the FVIIc-A assay, the measured FVIIc levels were higher than with the unmodified FVIIc-B assay, but still less than those achieved by the FVIIc-A assay. Therefore, no single reagent appeared to be responsible for the increased sensitivity of the FVIIc-B assay. It appears that the time allowed for incubation of the reagents with one another is important—specifically, the preincubation of test sample with the deficient plasma. In the FVIIc-A assay, this incubation is five minutes, whereas in the FVIIc-B assay it is 30 seconds. However, this variable by itself does not appear to explain the discrepancy entirely.

**DISCUSSION**

The data we have presented show that plasma from hemophilia A and B patients has a depressed factor VII activity state as measured by the FVIIc-B-VIIam ratio. The data further show that this depressed ratio results not only from a significantly decreased level of factor VII clotting activity, as measured by FVIIc-B, but also from a significantly increased factor VIIam level (Fig 1), which may reflect an increase in total factor VII concentration. Because the FVIIc-B results could have been caused either by an artifactual problem with the assay on hemophilic plasma or by activation of factor VII ex vivo in normal plasma, we performed a number of control experiments, none of which supported either possibility. We also demonstrated that the FVIIc-B method is more sensitive to cold activation of plasma factor VII than is our usual factor VII clotting assay (denoted FVIIc-A) (Table 2). Thus, our data support the conclusion that subjects with hemophilia have a decreased activity state of factor VII in vivo. A previous study reporting low factor VII levels (35% to 58%) in approximately one third of 43 patients with hemophilia B, regardless of the source of thromboplastin, probably detected the same phenomenon, but was interpreted as a combined inherited defect of factor VII and factor IX.17 The effect of such a decreased factor VII activity state is not known, although a similar decrease in plasma factor VII clotting activity in an otherwise normal individual is known to cause no clinical problems.18,19 It is possible, however, that when a lowered factor VII activity is combined with another hemostatic defect, as in hemophilia, the effect is less benign.

An examination of the coagulation cascade reveals a possible mechanism for the lowered factor VII activity state we have observed in hemophilia. We know that factor VII can be activated by factor Xa9,10,20 and that any generation of this enzyme is dependent on the presence of factors VIII and IX when tissue factor is absent. In addition, it is clear that factor VII in plasma is sensitive to very low levels of factor Xa.9 In hemophilic patients, then, a decreased generation of Xa could lead to a decreased activation of factor VII to VIIa. Slower pathways for factor VII activation, for example, via factor IXa or possibly thrombin,2,10 might also be affected, thereby accentuating the depression of factor VIIa. If we infer that normal or factor VII-deficient plasmas can generate an activating enzyme at levels sufficient to cause factor VII activation—for example, factor Xa—it is possible that the conditions of the FVIIc-A assay are more favorable for the detection of such activation than those of the VIIc-B assay.

Because the factor VII activity state as measured by the FVIIc-FVIIam ratio was lower than normal, the increased mean FVIIam level in hemophilic patients probably represents an increased concentration of unactivated factor VII, and therefore an increased total factor VII concentration. However, this deduction remains to be confirmed with a more specific, direct measure of total factor VII concentration, such as a radioimmunoassay for factor VII.21 The half-disappearance time of factor VII has previously been reported to be 300 minutes,19 whereas Seligsohn et al4 reported a factor VIIa half-disappearance time of only 144 minutes, suggesting that factor VIIa is more quickly removed from the circulation. If the turnover

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**Table 2. Activation of Plasma Factor VII in Glass at 4 °C**

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<tr>
<th>Subject No. 1</th>
<th>FVIIc-B (%)</th>
<th>FVIIc-A (%)</th>
<th>FVIIam (%)</th>
</tr>
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<tbody>
<tr>
<td>Fresh plasma</td>
<td>99.9</td>
<td>95.6</td>
<td>91.8</td>
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<tr>
<td>24-h plasma</td>
<td>266.1</td>
<td>130.6</td>
<td>93.9</td>
</tr>
<tr>
<td>Subject No. 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh plasma</td>
<td>96.1</td>
<td>96.6</td>
<td>104.6</td>
</tr>
<tr>
<td>24-h plasma</td>
<td>698.4</td>
<td>169.3</td>
<td>96.2</td>
</tr>
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Citrated plasma from two subjects on oral contraceptives was assayed for FVIIc-A, FVIIc-B, and FVIIam before and after storage in glass tubes for 24 hours at 4 °C. The experiment was performed identically on two separate occasions, and the results are shown as the average of the two independent determinations. Assays are described in Materials and Methods.
of factor VII to VIIa is slower in hemophilic patients, factor VII could accumulate, resulting in a higher total factor VII concentration than normal. Another possible mechanism for an increase in factor VII concentration could be factor VII synthesis regulated by a feedback mechanism dependent on factor VII activity rather than on total factor VII concentration. In this scheme, the lowered factor VIIa level would stimulate factor VII synthesis. The studies we have presented provide a clue to the possible importance in vivo of the relationship between the extrinsic and intrinsic pathways of coagulation. They suggest that a fraction of factor VII in normal blood does circulate as factor VIIa, and this in turn suggests that there is a low level of ongoing activation of the clotting system in the normal circulation.

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REFERENCES

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