RAPID COMMUNICATION

Measurement of Basal Levels of Factor VIIa in Hemophilia A and B Patients

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Previous results, presented in abstract form, indicate that replacement of thromboplastin with a mixture of phospholipid and truncated soluble tissue factor apoprotein results in a coagulation assay that can directly measure plasma factor VIIa levels without interference from zymogen factor VII (Atherosclerosis Thromb 11:1544a, 1991 [abstr]). We have exploited the specificity and sensitivity of such a factor VIIa specific coagulation assay to directly assess the in vivo relationship of factor VIII and factor IX on the production of factor VIIa levels under nonthrombotic and nonstimulatory conditions. Normal individuals (n = 20) were found to possess an average circulating factor VIIa level corresponding to 4.34 ± 1.57 ng/mL, or approximately 1% of their total factor VII antigen. Severe factor VIII deficient patients (n = 13) possessed a slightly lower but statistically significant (P < .01) decrease in their basal factor VIIa levels (2.69 ± 1.52 ng/mL), corresponding to ~80% of that observed in normal individuals. On the other hand, severe factor IX deficient patients (n = 7) were found to possess even lower levels of factor VIIa corresponding to 0.33 ± 0.15 ng/mL, or less than 10% of that observed in normal individuals. Measurement of total factor VII antigen levels shows that the variation in basal factor VIIa levels stems from differences in the degree of factor VII activation as opposed to differences in factor VII antigen levels. Our present data are consistent with the hypothesis that factor IXa is the principal in vivo activator of factor VII under basal conditions.

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MATERIALS AND METHODS

Bovine serum albumin (BSA) (fatty-acid free) was obtained from Sigma (St Louis MO). Bovine brain phospholipids (Thrombofax) were purchased from Ortho Diagnostics (Raritan, NJ). Factor VIIa deficient plasma (<1% factor VII antigen), obtained from a hereditary factor VII deficient patient, was generously provided by Dr Walter Kisiel (University of New Mexico, Albuquerque).

Recombinant human factor VIIa was purified from BHK cell culture medium as described previously. Recombinant soluble tissue factor apoprotein (residues 1 through 218) was purified from the culture media of transfected BL-21 (DE3) Escherichia coli cells by a combination of ammonium sulphate precipitation and S-Sepharose and Q-Sepharose ion exchange chromatography.

Total factor VII antigen levels were determined using a solid-phase double-antibody enzyme-linked immunosassay (ELISA) kit obtained from Novo Nordisk (Bagsvard, Denmark). This assay is a two-site monoclonal antibody assay in which a sandwich is formed between a solid-phase catching antibody, the factor VIIa:factor VIIa complex rapidly activates its protein substrates factors IX and X by limited proteolysis, which eventually leads to thrombin formation and a fibrin clot. Despite major advances into the biochemistry and in vitro behavior of the coagulation factors, little is known about the coagulation mechanism as it functions in vivo under basal (ie, in the absence of thrombosis or provocative stimuli) activation levels. The development of immunoassays for the activation peptides of human factor IX, human factor X, and human prothrombin have provided tools for the indirect measurement of coagulation activation. Based on these assay systems it has been suggested that the basal activity of the hemostatic system is primarily caused by factor VIIa:tissue factor function, which is responsible for the continuous generation of factor IXa, factor Xa, and thrombin. To investigate more closely the role of factor VIIa under basal conditions, we have used a factor VIIa specific coagulation assay to measure and directly compare the circulating factor VIIa levels in normal plasma as well as plasma from severe hemophilia A and B patients. In this report we provide direct evidence that basal factor VIIa levels in factor IX deficient patients are greatly reduced in comparison with normal individuals. These results are consistent with the hypothesis that factor IXa is the principal in vivo activator of factor VII under basal conditions.

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Submitted March 16, 1992; accepted April 18, 1992.

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subsequently incubated for 5 minutes at 37°C with 50 µL of bovine phospholipids (Thrombofax). Coagulation was then initiated by the addition of a 100-µL aliquot of 10 mmol/L TF1218 diluted in 12.5 mmol/L CaCl2/0.1 mol/L NaCl/0.05 mol/L Tris/1% BSA pH 7.4. Coagulation times were subsequently converted to factor VIIa concentration (nanograms per milliliter) by comparison to a standard curve constructed with varying concentrations (0.05 to 50 ng/mL) of purified recombinant factor VIIa diluted in TBS/BSA.

Collection and processing of blood samples. Venipunctures were performed atraumatically and blood samples drawn into citrated vacutainers. The citrated samples were centrifuged for 15 minutes at 1,200g after which time the plasma was removed with a plastic pipette and stored at −80°C. Normal plasma samples were collected from 10 fasting and 10 nonfasting individuals who had a negative history for bleeding as well as thrombosis and were not taking any medications at the time of sample collection. Patient samples were obtained from 13 severe hemophilia A and seven hemophilia B patients (<1% FVIII:C and <1% FIX:C). Patients were excluded from the study if they had received factor concentrates, cryoprecipitate, and/or antifibrinolytics within the previous 48 hours.

Informed consent. Informed consent was obtained from all patients before drawing of blood samples.

RESULTS

Results by Macik and Morrissey indicate that replacement of thromboplastin with a mixture of phospholipid and truncated soluble tissue factor apoprotein results in a coagulation assay that can directly measure plasma factor VIIa levels without interference from zymogen factor VII.10,11 Use of a similar assay system has enabled us to compare the circulating factor VIIa levels of normal individuals to that of individuals afflicted with hemophilia A or B.

In preliminary experiments we tested the effect of adding increasing amounts of purified recombinant factor VIIa on the coagulation time of factor VII deficient plasma in the presence of phospholipid and a soluble truncated form of recombinant tissue factor apoprotein (residues 1 through 218). By performing a log-log transformation of the coagulation data followed by a linear regression it was possible to construct a standard curve from which one could readily compare coagulation time with factor VIIa content (Fig 1). We found that this assay system could reproducibly measure factor VIIa levels over a wide range covering several orders of magnitude between 100 pg/mL to 1 ng/mL (data not shown).

In our initial experiments we measured the circulating factor VIIa levels of normal individuals under fasting and nonfasting conditions (n = 10 per group). The basal factor VIIa levels ranged from 2.21 to 7.85 ng/mL with an average of 4.34 ± 1.57 ng/mL (Fig 2). No statistical significance was observed between the plasma of fasting and nonfasting individuals. To determine the role of factor VIII or factor IX deficiency on basal factor VIIa levels, we also tested the functional factor VIIa level of 13 severe hemophilia A patients and seven severe hemophilia B patients. The hemophilia A patients possessed a basal factor VIIa level that varied between 1.32 and 6.24 ng/mL with an average of 2.69 ± 1.52 ng/mL (Fig 2). Thus, factor VIII deficiency significantly decreases (P < .01) basal factor VIIa levels to approximately 60% of that observed in normal individuals.

Repetitive measurement of basal factor VIIa levels in several hemophilia A patients over a time period of months indicates that basal factor VIIa levels remain essentially constant (data not shown).

Measurement of factor VIIa levels in hemophilia B patients shows a drastically lower factor VIIa level in comparison with both normal and hemophilia A patients (P < .01). The seven hemophilia B patients tested in this study were found to possess a circulating factor VIIa level that varied between 0.19 and 0.59 ng/mL with an average of

Fig 1. Factor VIIa standard coagulation curve. Varying amounts of recombinant factor VIIa were added to hereditary factor VII deficient plasma in the presence of phospholipid and soluble tissue factor apoprotein (residues 1 through 218). Coagulation was initiated by the addition of CaCl2 and the decrease in coagulation time measured on an automated coagulation apparatus as described in Materials and Methods.

Fig 2. Factor VIIa levels in the plasma of patients with severe factor VIII deficiency, severe factor IX deficiency, and normal controls. The horizontal bar represents the mean factor VIIa level in each group.
0.33 ± 0.15 ng/mL. Because the functional factor VIIa level in normal and hemophilic plasma was not influenced by prolonged incubation at room temperature or by such harsh treatment as repetitive freeze thawing, it is highly unlikely that any of the observed differences in factor VIIa levels are caused by ex vivo events.

In control experiments all plasma samples were subjected to factor VII antigen testing. As expected there was no significant (P > .1) difference in factor VII antigen levels between the hemophilia A (445 ± 93 ng/mL) and B patients (430 ± 89 ng/mL) and that of the normal control group (455 ± 72 ng/mL). Control experiments were also conducted to assess the recovery of factor VIIa activity after the addition of trace amounts of rFVIIa (5 to 10 ng/mL) to normal, factor VIII deficient, and factor IX deficient plasmas. As expected, there was virtually a 100% recovery of factor VIIa activity in each of the plasmas tested.

**DISCUSSION**

Measurement of the ratio of factor VII coagulant activity to amidolytic activity has previously been used as a crude measure of factor VIIa levels in hemophiliacs. Unfortunately this technique is rather imprecise because zymogen factor VII undergoes activation during the coagulation assay and thus contributes to the observed coagulant activity. However, this problem has recently been circumvented by the observation that a soluble form of tissue factor apoprotein, lacking the cytoplasmic and transmembrane domains, retains cofactor activity toward factor VIIa but is incapable of supporting the activation of zymogen factor VII. A study by Morrissey et al indicates that this soluble form of tissue factor can be used in a modified factor VIIa coagulation assay to specifically measure circulating factor VIIa levels. In the present study we have developed a similar assay system based on the use of a truncated tissue factor molecule spanning residues 1 through 218. We find that this assay system is exquisitely sensitive to trace amounts of factor VIIa and can reproducibly measure recombinant factor VIIa levels between 0.01 ng/mL to 1 mg/mL.

In this communication we have exploited the specificity and sensitivity of this factor VIIa specific coagulation assay to directly assess the in vivo levels of factor VIIa under nonthrombotic and nonstimulatory conditions. Previous attempts to assess the role of factor VIIa under basal conditions have centered on measuring the activation peptide that is released from factor IX on activation by factor X. In this regard it has recently been shown that factor VII deficient patients have reduced plasma levels of factor IX activation peptide, whereas FXI deficient individuals possess normal levels of factor IX activation peptide. In related experiments it has been observed that factor VII deficient patients also possess lower levels of factor X activation peptide as compared with factor VIII and factor IX deficient patients who possess essentially normal levels of factor X activation peptide. Although these data indicate that the generation of factor IXa and factor Xa under basal conditions is primarily caused by the action of factor VIIa:tissue factor, nothing is known about the role that these coagulation factors play on the activation of factor VII under basal conditions.

To investigate this question we initially measured the basal factor VIIa levels of normal individuals under fasting and nonfasting conditions. We found that normal individuals, regardless of fasting condition, possessed an average functional factor VIIa level corresponding to 4.3 ng/mL or approximately 1% of their total factor VII antigen (assuming an average factor VII antigen level of 400 ng/mL). To assess the in vivo role of factor VIII and factor IX on basal factor VIIa levels we also measured the factor VIIa levels of severe (< 1% activity) hemophilia A and B patients. Factor VIII deficiency was found to have a small but statistically significant effect on circulating factor VIIa levels, with patients on the average possessing a functional factor VIIa level of 60% of that observed in normals. Factor IX deficiency, on the other hand, was found to have a far greater effect with factor IX deficient patients on the average possessing a basal factor VIIa level corresponding to 10% of that observed in normal individuals.

Although previous in vitro studies indicate that factor Xa is a far more efficient activator of factor VII than factor IXa, our present data are consistent with the notion that factor IXa is responsible for basal activation of factor VII under in vivo conditions. Of course, it may be argued that the decrease in basal factor VIIa levels that we have observed in the hemophilia B patients is caused by a concomitant decrease in the functional levels of other serine proteases capable of activating factor VII, such as thrombin and factor Xa. However, this explanation is quite unlikely in view of previous observations that factor IX deficient and factor VIII deficient individuals possess normal levels of factor X activation peptide and prothrombin fragment 1,2. Precisely what role factor VIII plays in the activation of factor VII is less certain. However, one can readily envision that the reduced factor VIIa levels observed in the factor VIII deficient patients results from a concomitant decrease in basal factor IXa levels. Whether this hypothesis holds true or not will require measurement of basal factor IXa levels in factor VIII deficient patients.

In conclusion, we have developed a simple and highly specific coagulation assay that can reproducibly measure functional levels of factor VIIa in plasma. Using this assay system we have been able to assess the in vivo role of factor VIII and factor IX on the production of circulating factor VIIa levels under nonthrombotic and nonstimulatory conditions. Our results are consistent with the hypothesis that factor IXa is the principal in vivo activator of factor VII under basal conditions.

**ACKNOWLEDGMENT**

The authors thank Dr Lilian Tengborn, Sahlgren’s Hospital, Gothenburg, Sweden, and Dr Sam Schulman of Karolinska Sjukhuset, Stockholm, Sweden, for providing us with samples of factor IX deficient plasma. We also thank Dr Walter Kisiel, University of New Mexico, Albuquerque, for providing us with the hereditary factor VII deficient plasma and Carsten Christofferson for excellent technical assistance.
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Measurement of basal levels of factor VIIa in hemophilia A and B patients [see comments]

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