Factor IX Is Activated In Vivo by the Tissue Factor Mechanism

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Despite significant progress in elucidating the biochemistry of the hemostatic mechanism, the process of blood coagulation in vivo remains poorly understood. Factor IX is a vitamin K-dependent glycoprotein that can be activated by factor XIa or the factor VII-tissue factor complex in vitro. To investigate the role of these two pathways in factor IX activation in humans, we have developed a sensitive procedure for quantifying the peptide that is liberated with the generation of factor IXa. The antibody population used for the immunoassay was raised in rabbits and chromatographed on a factor IX-agarose immunoadsorbent to obtain antibody populations with minimal intrinsic reactivity toward factor IX. We determined that the mean level of the factor IX activation peptide (FIXP) in normal individuals under the age of 40 years was 203 pmol/L and that levels increased significantly with advancing age. The mean concentration of FIXP was markedly reduced to 22.7 pmol/L in nine patients with hereditary factor VII deficiency (factor VII coagulant activity less than 7%) but was not significantly different from normal controls in nine subjects with factor XI deficiency (factor XI coagulant activity less than 8%). These data indicate that factor IXa generation in vivo results mainly from the activity of the tissue factor mechanism rather than the contact system (factor XII, prekallikrein, high molecular-weight kininogen, factor XI). Our results may also help to explain the absence of a bleeding diathesis in many patients with deficiencies of the contact factors of coagulation.

THE COAGULATION system has classically been described as consisting of two distinct pathways, the intrinsic and extrinsic cascades, which converge to activate factor X.[1,2] The initiation of the intrinsic system is thought to occur with damage to the endothelium and the resulting exposure of subendothelial components such as collagen to the blood. The components of the intrinsic mechanism include the contact factors (factor XII, prekallikrein, high molecular-weight kininogen, factor XI) as well as factors IX and VIII. The extrinsic pathway is initiated when factor VII binds to tissue factor, an integral membrane glycoprotein that is expressed constituitively by subendothelial components of the vessel wall.[3] The factor Xa that is generated by either of the two cascades is then able to convert prothrombin to thrombin by binding to factor Va on activated platelets. The action of thrombin upon fibrinogen results in the formation of a fibrin clot.

During the last 35 years data have accumulated indicating that the intrinsic and extrinsic coagulation mechanisms do not function independently of each other. In the 1960s several groups of investigators provided circumstantial evidence that the components responsible for the initiation of the extrinsic cascade could activate factor IX in plasma.[4,5] However, it was not until 1977 that Osterud and Rapaport[6] clearly demonstrated that a mixture of partially purified factor VII and tissue factor could activate purified factor IX.

Human factor IX is a single-chain vitamin K-dependent glycoprotein with a mol wt of 57,000 that circulates in plasma as an inactive zymogen at a concentration of ~70 nmol/L.[7,8] The entire amino-acid structure of the human protein has been deduced from the cloning of its cDNA.[9] The activation of this component by factor XIa[7] or the factor VII-tissue factor complex[6] leads to the formation of factor IXa, which consists of two polypeptide chains joined by a disulfide bridge. During these reactions, peptide bonds at Arg_{145}-Ala_{146} and Arg_{180}-Val_{181} are cleaved, which releases a highly glycosylated 35 amino-acid activation peptide with a mol wt of ~11,000.[7]

There are currently no data regarding the relative contributions of the contact system and the factor VII-tissue factor mechanism to factor IXa generation in vivo. In this report we describe the development of a radioimmunoassay (RIA) for the activation peptide that is released from factor IX upon activation by factor XIa or the factor VII tissue-factor complex. The application of this technique to the study of patient populations with severe hereditary deficiencies of factor XI and factor VII indicates that the factor VII-tissue factor mechanism contributes in a dominant fashion to factor IX activation in humans.

METHODS

Purification of the factor IX activation peptide (FIXP). Human factor XIa, factor IX, and bovine factor XIa were obtained from Enzyme Research Laboratories (South Bend, IN). The FIXP was produced by activating human factor IX with bovine factor XIa (8% [wt/wt]) in the presence of 10 mmol/L $CaCl_2$ at 37°C. The reaction was terminated at 1 hour, and sodium dodecyl sulfate (SDS) gel electrophoresis documented the complete conversion of factor IX to factor IXa. The activation peptide was purified from this mixture by hydroxylapatite (Bio-Rad Laboratories, Richmond, CA) chromatography and reverse-phase high-pressure liquid chromatography (HPLC; Microsorb C_{18}, Rainin Instrument Co, Woburn, MA). The two steps were carried out in an identical fashion to those described for the isolation of the factor X activation peptide.[10] The FIXP eluted from the HPLC column at an acetonitrile concentration of 38% (vol/vol). The yield of FIXP was greater than 80% based upon the total amount of factor IX that had been activated. Amino acid

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analysis (Beckman 6500 Amino Acid Analyzer, Beckman Instruments Inc, Irvine, CA) was performed on a known volume of this preparation that had been subjected to acid hydrolysis. The peptide contained the same proportions of the relevant amino acids as that calculated from the known amino-acid composition of FIXP.[9] The molar amount of FIXP in the sample was obtained by dividing the nanomole sum of amino acids present by the number of substituents comprising the peptide.

Iodination of proteins. Radiolabeling of FIXP was carried out by the chloramine-T method of Greenwood et al[11] using 3 μg of peptide and 0.5 mCi of carrier-free $Na^{125}I$ or $Na^{131}I$ (New England Nuclear, Billerica, MA). After separation of the fragment from free iodide by Sephadex G-10 (Pharmacia Fine Chemicals, Piscataway, NJ) gel filtration, greater than 70% of the labeled material could be bound by high concentrations of antisera produced against the synthetic COOH-terminal peptide of FIXP. Human factor IX was radiolabeled as described above using 5 μg of protein and 0.2 mCi of $Na^{125}I$. The protein was separated from free iodide by Sephadex G-25 (Pharmacia Fine Chemicals) gel filtration.

Preparation of FIXP antisera. A peptide containing the COOH-terminal 16 residues of FIXP, NH_2-Leu-Asp-Asn-Ile-Thr-Gln-Gly-Thr-Gln-Ser-Phe-Asn-Asp-Phe-Thr-Arg-COOH, was synthesized using the solid-phase method of Merrifield.[12] The synthetic peptide was covalently linked to bovine serum albumin (BSA; Sigma Chemical Co, St. Louis, MO), and the conjugate was used to raise antisera in two rabbits. The methods were analogous to those employed by our laboratory to raise antisera to the factor X activation peptide.[10]

Antisera from individual bleeds were processed using an Affi-Gel Protein A column (Bio-Rad Laboratories) according to the manufacturer's instructions to obtain the immunoglobulin G (IgG) fractions. The specificity of each fraction was estimated by comparing the molar concentrations of factor IX and FIXP required to displace 50% of the immunoprecipitable ^{125}I-FIXP counts from the IgG preparation in the FIXP RIA described below. Each of the rabbits produced antisera in several bleedings that were approximately 100-fold more specific for the FIXP than factor IX on a molar basis. All data cited in this communication were obtained with an antiserum obtained from one of the rabbits after 3 months of immunization. To isolate an antibody population that had greater specificity toward FIXP, the IgG fractions were filtered in 0.05 mol/L sodium phosphate, 0.1 mol/L NaCl, pH 7.5, at 20 mL/h through a column of factor IX-Affi-Gel 15 (0.6 × 20 cm). This matrix was prepared by coupling 5 mg of human factor IX to 10 mL of "packed" Affi-Gel 15 (Bio-Rad Laboratories) as described by the manufacturer. Column effluents exhibiting significant absorbance at 280 nm were pooled and used to develop the FIXP RIA. These fractions contained 7% of the FIXP antibody initially present in the unfractionated antisera and demonstrated an additional 10-fold increase in specificity for the activation fragment as compared to factor IX.

FIXP RIA. This assay used a double-antibody approach. The initial reaction mixtures were composed of 50 μL of radiolabeled tracer (~10,000 cpm), 500 μL of unlabeled FIXP standards or unknown sample, as well as 100 μL of the specific antibody population. All of the reagents had been extensively diluted in Tris-buffered saline (0.05 mol/L Tris-HCl, 0.10 mol/L NaCl, 0.02% [wt/vol] sodium azide, pH 7.5, containing 1 mg/mL ovalbumin [Sigma Chemical Co]). The antibody concentration selected was able to precipitate 25% to 33% of the ^{125}I-FIXP when used in the absence of competing antigen. To act as a carrier in the second antibody separation, 4% (vol/vol) nonimmune rabbit serum was added to the solution containing the tracer. The tubes were mixed and then incubated at 4°C for 18 hours. Thereafter radiolabeled antigen bound to the antibody was separated from ^{125}I-labeled peptide. This was accomplished by adding 1 mL of 1% (vol/vol) goat antirabbit IgG (Scantibodies Laboratory, Lakeside, CA), 3.4% (wt/vol) polyethylene glycol (PEG) 6000 in Tris-buffered saline to each tube. The relative amounts of nonimmune rabbit sera (Scantibodies Laboratory) as well as goat antirabbit IgG were chosen to give the maximal precipitation of radiolabeled antigen. The tubes were centrifuged at 4°C for 20 minutes at 1800 g and washed once at 4°C with Tris-buffered saline. The resultant precipitates were quantified for ^{125}I counts. The detection limit of the technique is 0.02 nmol/L peptide. The mean slope and midpoint of the logit-log dose-response curves for 23 individual FIXP assays were -0.901 ± 0.07 and 0.394 ± 0.08, respectively.

Factor IX RIA. This assay was accomplished in a manner analogous to that described for the FIXP RIA except that the volume of unlabeled factor IX standard or unknown sample was only 50 μL. The factor IX antiserum (American Bioproducts Co, Parsippany, NJ) was diluted 50,000-fold to immunoprecipitate ~33% of the ^{125}I-factor IX counts in the absence of unlabeled antigen. The diluent for all reagents was 0.155 mol/L NaCl and 0.005 mol/L EDTA in 0.0225 mol/L sodium phosphate, pH 7.4, with 0.2% (wt/vol) sodium azide and 3.0% (wt/vol) BSA added. This assay is able to accurately measure factor IX down to a level of 0.05 nmol/L.

Collection and processing of blood samples. Venipunctures were performed atraumatically with 19- or 21-gauge butterfly infusion sets using a two-syringe technique. Blood samples for the FIXP RIA were drawn into plastic syringes preloaded with the following anticoagulant: 38 mmol/L citric acid, 75 mmol/L sodium citrate, 136 mmol/L dextrose, 6 mmol/L EDTA, 6 mmol/L adenosine, and 25 U/mL heparin (Elkins-Sinn, Inc, Cherry Hill, NJ). The ratio of anticoagulant to blood employed was 0.2:1.0 (vol/vol). Blood for routine coagulation studies was drawn into an anticoagulant containing 3.8% (wt/vol) sodium citrate; the ratio of anticoagulant to blood was 0.1:0.9 (vol/vol). After collection of blood samples, plasma fractions were obtained by centrifugation at 4°C for 15 minutes at 1600*g* and stored at −80°C before use.

Processing of plasma for FIXP RIA. The antibody fractions used in the FIXP RIA are approximately 1000-fold less reactive toward factor IX than the activation peptide. Thus a normal plasma factor IX level of 70 nmol/L might contribute as much as 0.07 nmol/L to the immunoreactive signal. We also observed that plasma constituents other than FIXP or factor IX contributed to a nonspecific basal signal in the assay. It was therefore necessary to develop a method by which the peptide could be extracted from plasmas of individual subjects. In this procedure larger proteins were precipitated by adding 0.1 vol of 7 mol/L perchloric acid to 0.9 vol of plasma and were then removed by centrifugation at 48,000 g for 20 minutes at room temperature. The supernatant fluid was decanted, 5 N NaOH was added to raise the pH to greater than 7, and 20% (vol/vol) trifluoroacetic acid (TFA) was admixed to lower the pH to less than 3. A butylsilane C_4 (6 mL) extraction column (J.T. Baker Inc, Phillipsburg, NJ) was prepared with 6 mL of absolute methanol containing 0.5% (vol/vol) TFA followed by 6 mL of 0.5% TFA. The sample was applied to this column, which was then washed with 12 mL of 0.5% TFA. The peptide was subsequently eluted with 4 mL of 75% (vol/vol) methanol containing 0.5% TFA into 12 ×75-mm test tubes. The contents of the tubes were evaporated to dryness overnight in a Savant Speed Vac Concentrator. Samples were individually reconstituted with 1.5 mL of 0.10 mol/L NaCl in 0.05 mol/L Tris-HCl, pH 7.5, containing 0.02% (wt/vol) sodium azide and 1 mg/mL ovalbumin. The specimens were then assayed by RIA for FIXP immunoreactivity.

The extraction of 6 mL of plasma from individual subjects enables us to concentrate the FIXP signal fourfold and lowers the detection limit of the assay technique to 5 pmol/L. A normal plasma control "spiked" with 0.5 pmol of FIXP as well as the same control plasma

are included with each set of samples to be processed. The results obtained on 10 occasions with a plasma pool obtained from normal donors indicate that the uncorrected basal level of the activation fragment is 211 ± 14 pmol/L. The recovery of the added peptide averaged 84.4% ± 9.9%. The extent of recovery was independent of the amount of FIXP added to the plasma or the volume of plasma used in the extraction process. As we have found that the average recovery of added FIXP in the extraction procedure is essentially constant, we have elected to not divide the values obtained by the fractional recovery. While the intra-assay coefficient of variation for the FIXP RIA was less than 5%, the within-day and between-day coefficients of variation for a plasma sample subjected to our extraction procedure were 8% and 12%, respectively.

Coagulation studies. Routine coagulation studies, including factor levels, were performed by standard laboratory methods.[13] The concentrations of factor VII antigen were determined by a solid-phase double-antibody enzyme-linked immunosorbent assay using a kit provided by Novo Alle (Bagsvaerd, Denmark).[14]

Informed consent. All clinical studies and informed consent procedures were approved by the Committee on Clinical Investigations, New Procedures and New Forms of Therapy of the Beth Israel Hospital.

Analysis of data. Estimation of relative immunoreactivity, computation of the slopes of the dose-response curves, as well as determinations of the various associated indices were obtained by a least-squares fit of the RIA results to a "four-parameter" model as described by Rodbard[15] and by Rodbard et al.[16] Statistical analyses of data were conducted by standard techniques.[17] In most instances the means are provided with associated standard deviations (SDs).

RESULTS

Antisera were raised in rabbits to a synthetic peptide containing the COOH-terminal sequence of FIXP. The IgG fractions were chromatographed on a factor IX-agarose immunoadsorbent to obtain antibody populations with minimal intrinsic reactivity toward factor IX. The preparations obtained were used to construct a double-antibody RIA, and the reactivity of factor IX was approximately 1000-fold less than that of FIXP on a molar basis (Fig 1).

The immunoreactivity of factor IX as well as the nonspecific contribution of other plasma constituents to our assay signal necessitated the development of a procedure by which the peptide could be extracted from plasma prior to assay. This was accomplished by precipitating plasma proteins including factor IX with perchloric acid and concentrating the activation fragment within the supernatant fraction with butylsilane (C_4) extraction columns. This methodology allows us to recover greater than 80% of the FIXP signal from plasma samples in a reproducible manner. We also determined the concentration of factor IX in reconstituted extracts of plasmas from normal individuals. The level was less than 0.4% of the initial plasma concentration of the zymogen. Thus, we can be certain that plasma factor IX does not contribute to the immunoreactive signal in the FIXP RIA. The validity of the measurements of factor IX activation in the plasma of normal subjects is supported by the fact that the RIA signal migrates on reverse-phase HPLC in a manner identical to that of the native peptide and can be quantitatively recovered (Fig 2).

During the quantification of plasma FIXP levels, we have ensured that in vitro factor IX activation is completely

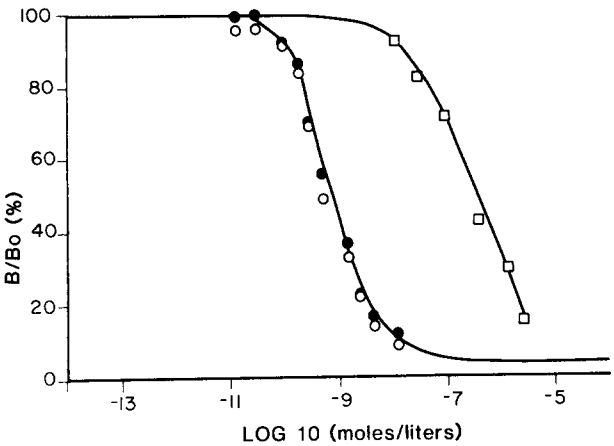

Fig 1. A comparison of FIXP (●) and factor IX (□) by RIA using an antibody population that had been chromatographed on factor IX-Affi-Gel 15. The ^{125}I-FIXP bound to the specific antibody in the presence of a given amount of competing antigen divided by the ^{125}I-FIXP bound to the specific antibody in the absence of competing antigen (B/B_0) is plotted against the $\log_{10}$ of the molar amount of competing antigen. The reactivity of factor IX was 1070-fold less than that of FIXP. To demonstrate that our antibody population is capable of detecting FIXP antigenic regions hidden within the factor IX molecule, a known amount of factor IX was completely converted to factor IXa and FIXP (○) by bovine factor XIa. The reaction conditions were identical to those used to purify the FIXP. Examination of this activation mixture with our RIA procedure confirmed that the theoretical amount of FIXP had been released.

suppressed after venipuncture. The solution used for this purpose contains ACD (citric acid, sodium citrate, dextrose), EDTA, adenosine (platelet inhibitor), and heparin (see Methods). The efficacy of this anticoagulant was demonstrated by immediately mixing blood samples from four normal donors with the above anticoagulant mixture. Purified human factor VIIa (final concentration, 0.5 μg/mL) or buffer was added to aliquots of each sample, and FIXP immunoreactivity was quantitated. The levels of this component in aliquots to which enzyme had been added were not significantly different from those in which buffer had been admixed (195 pmol/L ± 23 versus 191 pmol/L ± 38). Experiments in which human factor XIa (final concentration, 0.5 μg/mL) was the added enzyme gave similar results (208 pmol/L ± 89 versus 229 pmol/L ± 77). We also did not observe a significant difference in FIXP levels in normal individuals when blood samples were successively drawn into separate syringes containing the FIXP anticoagulant (195 pmol/L ± 77 versus 208 pmol/L ± 89).

To use the FIXP RIA to estimate the amount of factor IX that is activated in vivo, it is necessary to know the rate of clearance of the peptide. We have therefore undertaken studies of the metabolic behavior of FIXP in an animal model. The dog was chosen for these experiments, since we have previously obtained clearance rates for human prothrombin activation fragment F_{1+2} in this species that are similar to those in man.[18] To this end, ^{131}I-FIXP was infused as a bolus into a peripheral leg vein of the dog. At timed intervals blood samples were drawn into syringes containing the FIXP anticoagulant via a catheter placed in the jugular vein of the

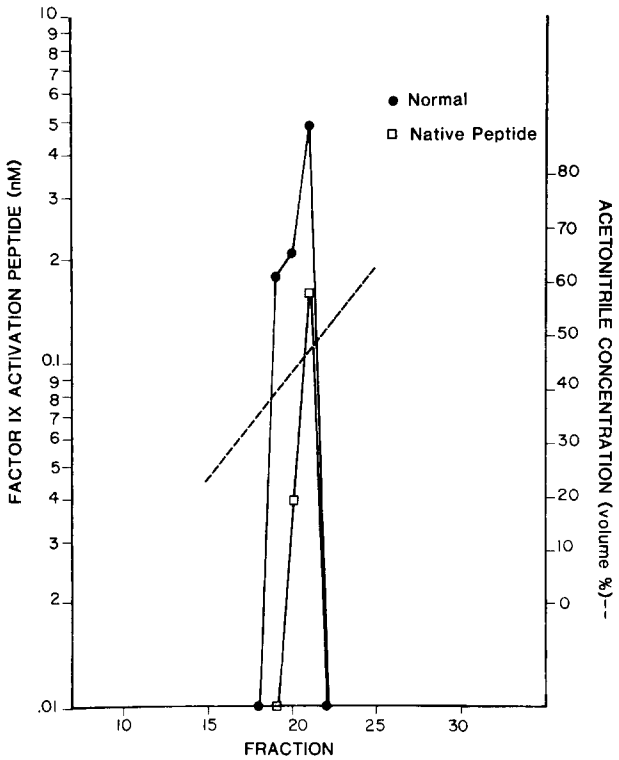

Fig 2. Analysis of the FIXP signal in normal plasma by reverse-phase HPLC. Plasma from a normal individual (6 mL) as well as a smaller volume of the same plasma "spiked" with native peptide were processed in the customary fashion (see Materials and Methods). The samples were reconstituted in 1 mL of 0.5% (vol/vol) TFA and injected onto a C_8 HPLC column (25 cm × 4.6 mm; Alltech Associates, Inc, Deerfield, IL). The mobile phase consisted of 0.5% (vol/vol) TFA, and the flow rate was 1.2 mL/min. The peptide was eluted with a linear gradient of acetonitrile in 0.5% (vol/vol) TFA. The contents of the fractions were evaporated to dryness and redissolved in 1.2 mL of RIA buffer. The FIXP RIA demonstrated that the peptide eluted at an acetonitrile concentration of ~45% (vol/vol) in both samples. Greater than 80% of the applied immunoreactivity was recovered in the peak fractions.

animal. Plasma radioactivity data were plotted against time. The ^{131}I-FIXP plasma radioactivity measurements can be described by a two-exponential curve, $C_1e^{-r1t} + C_2e^{-r2t}$.[19] The fractional breakdown rate, k_B (h^{-1}), was calculated from $[C_1/r_1 + C_2/r_2]^{-1}$.[20] The values for C_1, r_1, C_2, r_2, and k_B in two animals were 0.196, 0.221, 0.868, 3.53, and 0.883, and 0.263, 0.227, 0.742, 4.56, and 0.756, respectively. To verify that the iodination procedure did not alter the in vivo behavior of the activation fragment, turnover studies with unlabeled native-activation peptide were performed in each dog within 5 days of the ^{131}I-FIXP infusion study. Samples were obtained at various time points and assayed immunologically for FIXP. These experiments indicated that the iodination procedure did not substantially alter the in vivo behavior of the radioactive species (data not shown).

The extent of factor IX activation was first established in normal individuals. This population consisted of individuals who gave a negative history for bleeding as well as thrombosis and were not taking any medications at the time of sample collection. FIXP levels in 17 control males and females under the age of 40 years (median age 29 years, range 20 to 39 years) ranged between 131 and 284 pmol/L with a mean of 203 ± 41 pmol/L (Fig 3). The concentration of FIXP was also determined in the blood of 69 healthy males between the ages of 40 and 80 years. These individuals were participants of the Normative Aging Study of the Veterans Administration Outpatient Clinic, Boston, MA. The Normative Aging Study is a longitudinal investigation of aging phenomena in males,[21,22] and individuals with coexistent medical conditions (ie, venous or arterial thrombotic disease, hypertension, diabetes, cancer, renal disease, liver dysfunction, etc) that might cause alterations in hemostatic system activity as measured by our RIA were excluded from our population. The mean FIXP level in these men increased as a function of age (Table 1). Statistical analysis demonstrated that plasma measurements of FIXP correlate strongly with age within this cohort ($r = .337$, $P < .01$).

We next determined the FIXP levels in the blood of patients with hereditary deficiencies of factor IX, VII, or XI (Fig 3). All were asymptomatic at the time of sampling. Nine patients with hereditary factor IX deficiency (factor IX coagulant activity levels of ≤3%) and a severe bleeding diathesis (median age 28 years, range 13 to 63 years) were evaluated. The mean FIXP value of 21.2 ± 9.7 pmol/L in this group was reduced approximately 10-fold as compared with controls under the age of 40 years.

We then analyzed the extent of factor IX activation in nine factor VII-deficient subjects from eight kindreds (median age 27 years, range 14 to 76 years); eight of the subjects had

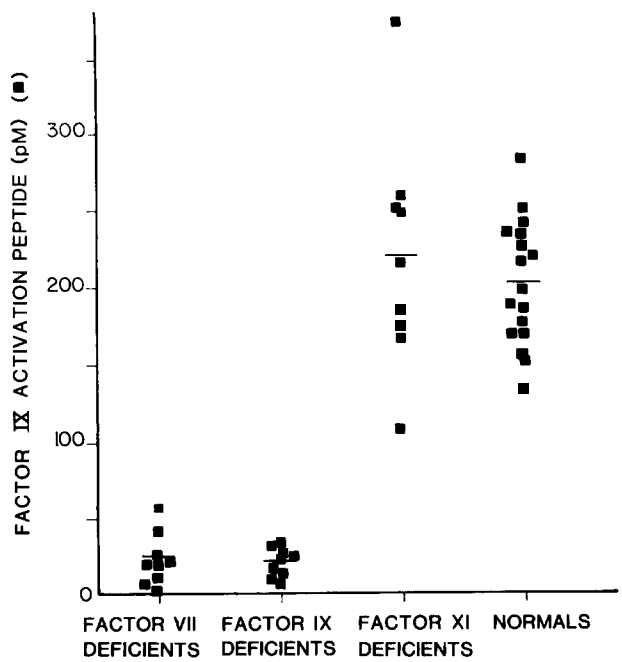

Fig 3. Factor IX activation peptide levels in the plasma of patients with hereditary deficiencies of factor VII, factor IX, or factor XI and normal controls less than 40 years of age. Horizontal bars represent the mean value of the parameter in each group of subjects.

Table 1. Levels of the Factor IX Activation Peptide in Healthy, Aging Males

Age Group (yrs)	No. of Subjects	Mean Value ± SD (pmol/L)	Range
41-50	23	216 ± 56	134-355
51-60	14	228 ± 74	112-399
61-70	15	268 ± 84	175-456
71-80	17	274 ± 70	157-394

a prior history of a mild-to-moderate bleeding diathesis. The measurements of factor VII coagulant activity were less than 7% of normal in each of these individuals, and the factor VII antigen measurements were also markedly reduced. The mean level of FIXP in this group of patients was markedly reduced to 22.7 ± 16 pmol/L ($P < .0001$ as compared with normal controls under the age of 40 years). While three of these subjects were over the age of 40 years, it should be noted that comparison of the FIXP levels in the entire factor VII-deficient cohort with a perfectly age-matched control group would serve to further magnify the difference between these two populations.

Nine factor XI-deficient patients from eight families were investigated (median age 33 years, range 15 to 76 years); only two of these individuals had experienced bleeding episodes that occurred in association with surgical procedures. All had factor XI coagulant activity levels of less than 8% of normal. However, in contrast to our results in factor VII-deficient individuals, the mean concentration of the activation fragment in these subjects was 221 ± 74 pmol/L ($P > .1$ as compared with normal controls). Three of these individuals were over the age of 40 years.

DISCUSSION

Our laboratory has previously developed sensitive RIAs for the fragments that are liberated with the activation of prothrombin[23,24] and factor X.[10] The specific antibody populations used in these assays recognize antigenic sites in the COOH-terminal region of the activation peptide that are hidden in the parent zymogen. We have employed this approach to obtain an IgG preparation for the peptide that is generated when factor IX is activated by factor VII/VIIa-tissue factor or factor XIa. This antibody population was then used to develop a sensitive and specific assay procedure to quantify the levels of this component in human plasma. We determined that the normal plasma FIXP level is ~200 pmol/L in subjects under the age of 40 years and that the levels of this component increase significantly with advancing age.

Our studies of the FIXP clearance rate in an animal model permit us to estimate the extent to which physiologic activation of the coagulation mechanism contributes to the catabolism of factor IX in the circulation. In a steady state, the rate of FIXP production, F, must equal the breakdown rate, B. Under these conditions: F (nmol/h) = B (nmol/h) = $k_B \times V \times C$, where V is the plasma volume of the patient, k_B is the fraction of plasma FIXP broken down per hour in a steady state, and C is the ambient FIXP concentration.[25] Using this equation, we calculate that a normal 70-kg individual (plasma volume ~ 2.8 L, $k_B \sim 0.8$) with an ambient FIXP value of 0.2 nmol/L would generate 0.45 nmol/h of this fragment. While the catabolic rate of factor IX in humans has not been rigorously determined, we are able to estimate that this value is 13 nmol/h. This was calculated based on a known prothrombin-catabolic rate of 0.104 μmol/h[26] and assumes that the plasma concentration of factor IX is one twentieth that of prothrombin, while its fractional breakdown rate is 2.5 times that of the latter zymogen. Thus the rate of production of FIXP is equivalent to 3.5% of the normal daily catabolism of factor IX on a molar basis. It is interesting to note that we previously calculated that the activation of factor X accounts for only 1.8% of the normal daily catabolism of this zymogen.[10]

To assess the contributions of the contact factors and the factor VII-tissue factor mechanism to factor IX activation in vivo, we have determined the plasma concentrations of FIXP in patients with various hereditary clotting-factor deficiencies. Individuals with a severe deficiency of factor IX and, more interestingly, factor VII exhibited a striking reduction in the levels of this activation fragment as compared with controls under the age of 40 years. These data are markedly different from a group of factor XI-deficient patients who had a mean concentration of FIXP that was not significantly different from normal subjects.

Based upon studies using immunologic markers of hemostatic system activation, there is evidence that the coagulation mechanism is continuously active in vivo and is held in check by natural anticoagulant mechanisms.[27] The development of the FIXP RIA has allowed us to provide the first direct evidence that factor IX can be activated in vivo by the factor VII-tissue factor complex and that the contact system is relatively quiescent in normal subjects. This activation may occur outside of the vasculature due to the normal passage of coagulation proteins through endothelial cells[28] into tissues that constituitively express tissue factor activity.[29] Alternatively there may be small amounts of tissue factor on the endothelium that cannot be demonstrated by current methodologies but are able to mediate the factor VII/VIIa-dependent activation of factor IX. The fact that the tissue factor mechanism is active under normal conditions also suggests that this system is important in mediating the response to provocative stimuli that are capable of triggering pathologic thrombosis. We also cannot exclude that factor XIa may be important in activating factor IX before thrombotic events or at sites of hemostatic plug formation.

The above observations may help to explain the hemorrhagic events that are encountered in most patients with severe factor VII deficiency[30,31] and the relative absence of such episodes in many individuals with factor XI deficiency.[32,33] People with deficiencies of the other contact factors do not exhibit bleeding diatheses, and we anticipate that they will have normal FIXP values. It is also possible to speculate that the occasional patient with factor XI deficiency who experiences excessive bleeding will turn out to have reduced activity of the tissue factor mechanism or some other as yet undefined hemostatic defect.

Finally, the Northwick Park Heart Study[34] has reported that factor VII coagulant-activity measurements correlate

strongly with the development of coronary thrombosis in middle-aged men. Given the sensitivity of the FIXP RIA to alterations in the in vivo activity of the factor VII-tissue factor mechanism, this technique may prove to be particularly valuable as a marker for identifying those individuals at highest risk for the subsequent development of thrombotic phenomena.

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