Congenital Deficiency of Blood Clotting Factors II, VII, IX, and X

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A patient congenitally deficient in factors II, VII, IX, and X has been further investigated after a follow-up of 15 yr. At birth, these factors, when determined by clotting assays, were undetectable. Following therapy with vitamin K₁, the clotting activity of these factors rose but never exceeded 18% of normal. Immunologic assays revealed much higher levels of these factors than did clotting assays, thus suggesting that the vitamin-K-dependent factors were present in abnormal forms. Two-dimensional crossed immunoelectrophoresis showed that at least two forms of prothrombin were present in the patient’s plasma. One form was similar to normal prothrombin; the other had the same mobility as acarboxyprothrombin. In addition, the majority of this fast-migrating peak was not adsorbable onto insoluble barium salts. These observations suggested that some molecules of the patient’s prothrombin lacked the normal complement of gamma-carboxyglutamic acid residues. This observation was confirmed by a specific assay for gamma-carboxyglutamate. Since malabsorption of vitamin K, warfarin intoxication, and hepatic dysfunction were excluded as causes of this patient’s syndrome, this rare congenital abnormality could represent either a defective gamma-carboxylation mechanism within the hepatocyte or faulty vitamin K transport.

It has been known for many years that vitamin K is necessary for the complete synthesis of prothrombin (factor II) and factors VII, IX, and X. Vitamin K is also necessary for the production of protein C in the bovine species and protein S in man. Recent investigations have shown that vitamin K acts at a postribosomal level in the hepatocyte to modify precursor forms of these factors. Thus, vitamin K, in the presence of a carboxylating enzyme, hepatic microsomes, O₂, and CO₂, converts glutamic acid residues on the amino-terminal regions of precursor forms of prothrombin and factors VII, IX, and X to gamma-carboxyglutamic acid residues. These gamma-carboxyglutamic acid residues are necessary for calcium-dependent phospholipid binding by the vitamin-K-dependent clotting factors and are prerequisites for normal blood coagulation.

Whereas defective gamma-carboxylation of glutamic acid residues occurs following therapy with coumarin drugs, a congenital abnormality resulting in defective gamma-carboxylation of the vitamin-K-dependent factors has hitherto not been reported.

We have reevaluated a unique 15-yr-old female who has congenital deficiency of
the vitamin-K-dependent blood clotting factors. She was initially seen at the age of 3 mo with multiple bruises and a history of numerous hemorrhagic episodes. Vitamin K deficiency due to malabsorption, liver disease, and warfarin intoxication was excluded, both at the time of initial presentation and on several subsequent occasions. Based on current studies, it appears that this patient may have a defect in the gamma-carboxylation mechanism that results in the production of immunologically recognizable factors II, VII, IX, and X that lack the full complement of gamma-carboxyglutamic acid residues and therefore lack coagulant activity.

MATERIALS AND METHODS

Patient

The patient has been previously described. When she was initially seen it was determined by assay of her plasma for bishydroxycoumarin and warfarin that she had received no anticoagulant drugs. Subsequent assays for these drugs have also been negative. At the age of 6 mo the patient was observed for 2 wk in the hospital, at which time it was ascertained that she was not receiving vitamin K antagonists surreptitiously. In addition, studies at that time revealed no evidence for malabsorption or liver disease. Subsequent to the initial report, the patient has been followed periodically for 15 yr. During this time she has required daily vitamin K supplements. Initially vitamin K was administered parenterally, but subsequently oral doses have been shown to be efficacious. However, even with massive intravenous doses of vitamin K, the patient's K-dependent factors never returned to normal, although the prothrombin time decreased from more than 100 sec to 20–25 sec. Withdrawal of vitamin K resulted in prompt return of bleeding and a marked decrease in the clotting activities of prothrombin and factors VII, IX, and X. Studies on parents of the patient showed normal levels of factors II, VII, IX, and X.

Plasma

Normal human blood was collected from 10 normal donors into 3.2% trisodium citrate (8 parts blood to 1 part citrate). After centrifugation the plasma was pooled and stored at −70°C. The patient's plasma was prepared in the same manner.

Reagents

Sodium chloride, sodium citrate, kaolin, calcium lactate, diethylbarbituric acid, tris(hydroxymethyl)aminomethane, sodium azide, benzene, monobasic and dibasic sodium phosphate, and barium chloride were purchased from Fisher Scientific, Fair Lawn, N.J. Imidazole, acrylamide, and cyanogen bromide were purchased from Eastman Kodak, Rochester, N.Y. Calcium chloride, methanol, glacial acetic acid, and formic acid were obtained from Mallinckrodt, St. Louis, Mo. Sephadex G-100 and Sepharose 4B were obtained from Pharmacia Fine Chemicals, Piscataway, N.J., and Uppsala, Sweden. Alumina C–γ gel was purchased from Calbiochem, La Jolla, Calif. Borosilicate glass clotting tubes (10 × 75 mm) were purchased from Arthur H. Thomas, Philadelphia, Pa. Agarose and Coomassie brilliant blue were from Bio-Rad Laboratories, Richmond, Calif. Simplastin was purchased from General Diagnostics, Morris Plains, N.J. Thrombofax was from Ortho Diagnostics, Raritan, N.J. Dansyl chloride and the venom from Echis carinatus were obtained from Sigma Chemical, St. Louis, Mo. Staphylococcalase was purchased from Diagnostica Stago, Asnieres, France. Canine factor V and fibrinogen were obtained from platelet-poor canine oxalated plasma that was exhaustively treated with barium sulfate and then raised to 25% saturation with (NH₄)₂SO₄ (to obtain fibrinogen-rich material) and then to 33% (to obtain factor-V-rich material).

Normal Human Prothrombin

Highly purified normal human prothrombin was a by-product of our factor IX purification procedure employing heparin agarose chromatography as the last step.

Heterologous Antibodies to Normal Human Prothrombin

Antibodies to normal human prothrombin were raised in two rabbits by four weekly injections of 0.25 mg of pure prothrombin and an equal volume of complete Freund's adjuvant. The rabbits were then bled
at weekly intervals, beginning 1 wk after completion of the immunization. The rabbit serum was heated at 56°C for 30 min and adsorbed with one-tenth volume of alumina C-γ gel. After centrifugation, the supernatant was dialyzed against 0.01-M sodium phosphate buffer (pH 7.0), and the dialysate was applied to a prothrombin-Sepharose affinity column that was prepared by coupling normal prothrombin to cyanogen-bromide-activated Sepharose 4B. Antiprothrombin antibodies were eluted with 0.1-M glycine-HCl (pH 2.8), then immediately brought to pH 7.0 by the dropwise addition of dilute NaOH.

**Purification of the Patient’s Prothrombin**

Because there were only limited quantities of the patient’s plasma, the patient’s prothrombin was prepared from small amounts of citrated plasma. Ten milliliters of the patient’s plasma was stirred with 1 ml of 1-M BaCl₂ and centrifuged. The supernatant containing nonadsorbable prothrombin was dialyzed against 0.01-M sodium phosphate buffer (pH 7.0) and applied to an antiprothrombin antibody affinity column (1.5 X 4.0 cm) that was prepared by coupling the purified antibody to cyanogen-bromide-activated Sepharose 4B. The prothrombin was eluted with 0.1-M glycine-HCl buffer (pH 2.8) and immediately raised to pH 7.0 with dilute NaOH. The eluate was then concentrated and subjected to gel filtration on a Sephadex G-100 column (0.35 x 8.5 cm). The purification was monitored immunologically by the Ouchterlony double-diffusion technique. The results showed a single precipitin line when the preparation was tested against an antiprothrombin antibody, against anti-whole-human-plasma, and against antisera to the barium citrate eluate of normal human plasma.

**Determination of Gamma-Carboxyglutamic Acid in Patient and Normal Prothrombin**

Gamma-carboxyglutamic acid residues were assessed in the purified prothrombin from both normal plasma and the patient’s plasma using thin-layer polyamide chromatography. Purified prothrombin was hydrolyzed for 24 hr at 100°C in 2-M KOH. The resulting amino acids were labeled with dansyl chloride and subjected to two-dimensional chromatography on thin-layer polyamide sheets using H₂O-formic acid (100:1.5 v/v) in the first dimension and benzene-acetic acid (9:1 v/v) in the second dimension. Labeled amino acids could be detected under short-wave ultraviolet light (λ = 254 nm). A synthetic monomeric gamma-carboxyglutamic acid was prepared according to the methods of Boggs et al. It was then dansylated, subjected to chromatography as described previously, and used as a control to detect a similar amino acid in normal prothrombin and in the patient’s prothrombin.

**Coagulation Assays**

Blood clotting factors VII, IX, and X were assayed in one-stage tests using as substrate platelet-poor plasma obtained from patients congenitally deficient in the respective factors. Prothrombin coagulant activity was measured with the one-stage method, the two-stage method, staphylocoagulase, and venom from *E. carinatus*. For the latter method, normal plasma and the patient’s plasma diluted 1:7, 1:14, 1:21, and 1:28 in imidazole-HCl buffer (pH 7.2) were tested. Two milligrams of *E. carinatus* venom were dissolved in 1 ml of normal saline; 10 μl of this solution were added to 0.2 ml of the plasma dilutions and incubated for 10 min at 37°C. Two-tenths milliliter of canine fibrinogen was then added, a stop watch was started simultaneously, and the tubes were tilted and observed for the appearance of a clot.

**Immunologic Procedures**

Antibody neutralization was monitored in clotting assays as follows: Factor IX neutralization assays were conducted by a previously described method with a well-characterized specific human inhibitor of factor IX. Factor X neutralization was a modification of the factor IX neutralization assay using a heterologous antibody to factor X. Prothrombin neutralization was conducted using a heterologous antibody to factor II in a two-stage assay to detect residual prothrombin activity.

Radial immunodiffusion was performed in 1% agarose in 0.1-M phosphate-buffered saline at pH 7.0. Electroimmunoassays were performed with agarose containing a specific heterologous antibody to prothrombin. Crossed immunoelectrophoresis was conducted in 0.075-M tris-barbital buffer (pH 8.6) containing 2-mM calcium lactate at 4°C in both directions.

**RESULTS**

Clotting factors not dependent on vitamin K for synthesis have been normal throughout the patient’s life, as shown in Table 1. Factor V was initially 57% of
normal, but has subsequently been 100% of normal. The patient's vitamin-K-dependent clotting factor levels are shown in Table 2. Note that factors II, VII, IX, and X were undetectable before the patient was treated with vitamin K. Administration of large doses of oral vitamin K resulted in an increase in these factors not exceeding 18% of normal. However, despite massive doses of parenteral vitamin K, the patient’s vitamin-K-dependent factors never reached normal levels.

To determine if the vitamin-K-dependent factors were actually as low as indicated by clotting assays, factors II, IX, and X were measured by immunologic assays using antibody neutralization tests. The results shown in Table 3 indicate that prothrombin is present in a concentration 57% of normal, factor IX in a

*Appropriate monospecific antibody not available.
concentration 100% of normal, and factor X in a concentration 55% of normal. Factor VII was not measured immunologically because an appropriate antibody was not available for this purpose. These data strongly suggested that altered forms of the vitamin-K-dependent factors were present in the patient’s plasma, forms that retained immunologic activity but lacked coagulant activity.

To explore this possibility further, the prothrombin content of the patient’s plasma was measured by several techniques and compared with that of normal pooled human plasma and of plasma obtained from a patient on warfarin therapy.

Fig. 1. Crossed immunoelectrophoresis: (A) patient’s plasma, (B) plasma from a patient on warfarin therapy, (C) normal pooled plasma. The first dimension was run for 3.5 hr at 10 v/cm, and the second dimension was run overnight at 20 v/cm. Both dimensions were run in 1% agarose containing 2-mM calcium lactate. In the second dimension the agarose contained a specific antibody to prothrombin.
As shown in Table 4, when using physiologic activators, i.e., one- and two-stage clotting assays, the prothrombin clotting activity was found to be very low (less than 10% of normal). In contrast, when using nonphysiologic activators such as \textit{E. carinatus} venom and staphylocoagulase, the levels of prothrombin were found to be 47% and 40% of normal, respectively. Immunologic techniques using a specific heterologous antiprothrombin antibody gave results similar to those obtained using nonphysiologic activators. Similar results were obtained when assaying plasma from a patient receiving long-term anticoagulant therapy with warfarin. After adsorption of the patient's plasma by barium citrate, the supernatant exhibited prothrombin activity when using nonphysiologic activators, and these results were corroborated by immunologic assays.

When subjected to crossed immunoelectrophoresis (Fig. 1), the patient's plasma exhibited at least two peaks of prothrombin (Fig. 1a). The smaller of the two peaks had the same electrophoretic mobility as normal prothrombin (Fig. 1c), whereas the major peak had the same electrophoretic mobility as the acarboxyprothrombin found in plasma from a patient on warfarin therapy (Fig. 1b). These data suggest that most of the patient's prothrombin existed in the acarboxy form.

The patient's plasma was then adsorbed with barium citrate, which usually removes all normal prothrombin, but not acarboxyprothrombin, in warfarin plasma. Resulting supernatant plasma was tested by crossed immunoelectrophoresis for residual prothrombin (Fig. 2). The peak corresponding to normal prothrombin was

\begin{figure}[h]
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\caption{Crossed immunoelectrophoresis: (A) patient's plasma after adsorption by barium citrate, (B) normal pooled plasma control. The conditions are the same as those described in Fig. 1.}
\end{figure}
removed by barium citrate, whereas most of the peak with the same electrophoretic mobility as acarboxyprothrombin remained. The barium citrate cake resulting from adsorption of the patient’s native plasma was eluted and then subjected to crossed immunoelectrophoresis (Fig. 3). A peak of prothrombin with the same electrophoretic mobility as normal prothrombin could be found, but in addition a faster-migrating peak was also found, thus indicating that at least some of the abnormal prothrombin was adsorbed by barium salts.

A more direct method was used to determine the content of gamma-carboxyglutamic acid residues in the patient’s purified nonadsorbable prothrombin. Alkaline hydrolysis and two-dimensional thin-layer chromatography of the patient’s prothrombin and normal prothrombin were performed. The dansylated amino acids from the patient’s prothrombin and normal prothrombin were compared to each other and to a standard synthetic monomeric gamma-carboxyglutamic acid. The migration of the synthetic monomeric gamma-carboxyglutamic acid in this experiment is shown in Fig. 4c. A fluorescent spot corresponding to the gamma-carboxyglutamic acid control was found in normal prothrombin even when normal prothrombin was diluted to about the same antigenic concentration as the patient’s prothrombin (Figs. 4b and 4d). However, when the patient’s prothrombin was chromatographed in this system (Fig. 4a), no fluorescent spot corresponding to
gamma-carboxyglutamic acid was seen. This technique is sensitive to a prothrombin gamma-carboxyglutamic acid content 1% of normal.

DISCUSSION

A unique patient with congenital deficiency of factors II, VII, IX, and X has been evaluated. Even after therapy with massive doses of vitamin K, either parenterally or orally, her plasma showed greatly reduced levels of the vitamin-K-dependent factors when measured by clotting assays. However, when immuno-logic techniques were employed to measure these factors, much greater quantities of the vitamin-K-dependent clotting proteins were found. Since vitamin K deficiency due to malabsorption, liver disease, and warfarin ingestion had been excluded by appropriate biochemical and spectrophotometric tests, the patient’s partial response to parenteral and oral vitamin K suggested a defect in vitamin K.
metabolism. Defective vitamin K metabolism could lead to the production of blood clotting factors deficient in gamma-carboxyglutamic acid. Gamma-carboxyglutamic acid residues are normally found on the amino-terminal end of molecules of the vitamin-K-dependent factors and are necessary for these factors to bind not only to calcium but also to insoluble barium salts. Calcium binding is necessary for the subsequent binding of these factors to phospholipids or specific receptors on the platelet surface. In the absence of calcium-dependent phospholipid binding, the vitamin-K-dependent factors do not participate normally in blood coagulation.

The patient's prothrombin after vitamin K treatment was found to be 3.5% and 7% of normal when measured using clotting assays dependent on calcium binding. However, when the patient's prothrombin was assessed by nonphysiologic activators, which do not depend on the presence of gamma-carboxyglutamic acid residues for the generation of thrombin, the level of prothrombin was found to be 40% and 47% of normal. Similarly, immunologic assays of prothrombin, factor IX, and factor X were 57%, 100%, and 55% of normal. These data strongly suggest that factors II, IX, and X in the patient's plasma lacked the full complement of gamma-carboxyglutamic acid residues. This hypothesis is supported by the finding that on crossed immunoelectrophoresis in the presence of calcium ions the patient had at least two peaks of prothrombin, one corresponding in mobility to normal prothrombin and the other corresponding to a more rapidly migrating peak found in the plasma of patients treated with warfarin. The rapid peak in the patient's plasma is essentially not adsorbable onto barium salts, again suggesting that the patient's prothrombin contained a decreased complement of gamma-carboxyglutamic acid residues. This hypothesis was substantiated with the observation that amino acids from the patient's purified nonadsorbable prothrombin contained no detectable gamma-carboxyglutamic acid residues when tested by a qualitative technique employing thin-layer chromatography of dansylated amino acids.

In addition to normal prothrombin and acarboxyprothrombin, the patient's plasma contains another form of prothrombin that behaves on crossed immunoelectrophoresis, in the presence of calcium ions, as a fast-migrating peak but has the ability to be adsorbed onto insoluble barium salts. These results suggest that the patient's prothrombin is heterogeneous with respect to the content of gamma-carboxyglutamic acid residues. This hypothesis is supported by the findings of Esnouf and Prowse, who reported different degrees of carboxylation of prothrombin molecules obtained from patients on warfarin therapy.

Since even with immunologic techniques the patient's plasma contained only about 50% of the prothrombin found in normal plasma, the question arises why prothrombin deficient in gamma-carboxyglutamic acid residues is not present in amounts approaching 100% of normal. The answer to this question is not known, but it is possible that acarboxyprothrombin has a shorter biologic half-life than normal prothrombin, as has been suggested by Lavergne and Josso. Thompson has suggested a similar explanation for decreased immunologic levels of acarboxy factor IX found in patients on warfarin therapy.

Gamma-carboxyglutamic acid as a constituent of the vitamin-K-dependent clotting factors has been described by Stenflo, Nelsestuen et al., and Magnusson et al. Later, Hauschka et al. described the presence of these same residues in bone and in the kidney. Their results suggest that the gamma-carboxyglutamic acid in proteins other than the blood clotting factors is also apparently vitamin-K-dependent. It should be noted, however, that our patient had normal renal
FACTORS II, VII, IX, AND X

function and normal skeletal development, even though her vitamin-K-dependent clotting factors had been decreased since birth and during the subsequent 15 yr of follow-up. Thus if the patient’s defective vitamin K metabolism had affected her skeletal or renal development, the defects were either obscured by vitamin K therapy or not detected in these tissues, our end points being currently too crude. It is also possible that defects in the renal or skeletal system will be manifest only after the patient gets older.

Although vitamin K deficiency per se and vitamin K antagonism have been excluded in this patient, the precise defect in vitamin K metabolism is yet to be clarified. There could be an abnormality in transport of vitamin K in the blood. However, a carrier protein for vitamin K has not been identified. A second possibility is that vitamin K may not be handled normally in the liver. Suttie et al. have suggested that vitamin K, when it enters the hepatocyte, is obligatorily converted to vitamin K, epoxide by an epoxidase system. Vitamin K, epoxide is then converted to reduced vitamin K, by a specific reductase system in the hepatocyte before it can act as a co-factor in the carboxylating mechanism. We have been unable to exclude in this patient defects of the epoxidase or reductase systems. Another possibility is that vitamin K is metabolized normally within the hepatocyte but that it does not function as a co-factor for gamma-carboxylation because of a defective carboxylating mechanism. Suttie et al. have demonstrated that the gamma-carboxylation of glutamic acid residues in the hepatocyte requires hepatic microsomes, reduced vitamin K, a carboxylating enzyme, O₂, and CO₂. It seems unlikely that the patient’s hepatic microsomes are defective, but it is possible that the patient has a congenitally defective carboxylating mechanism for the vitamin-K-dependent clotting factors. Evaluation of the patient’s own hepatic microsomes would be necessary to test this hypothesis, but this experiment is not possible at the present time.

Nevertheless, it is clear that this unique patient has congenital deficiency in the clotting activity of factors II, VII, IX, and X, and to our knowledge she is the only such patient reported with this defect. We have not yet tested this patient for the presence of protein S, which is another vitamin-K-dependent factor found in humans. The function of this protein is not yet known. The current studies demonstrate that the defect in this patient is due to synthesis of clotting factors lacking the normal complement of gamma-carboxyglutamic acid residues. Since carboxylation of glutamic acid residues is dependent on vitamin K, it follows that there is some type of interference in metabolism of vitamin K, either by defective transport in the blood or by defective intrahepatic epoxidase, reductase, or carboxylase systems. While the latter hypothesis may be more attractive in view of recent knowledge, it is not yet possible to distinguish among these possibilities.

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