In Vivo Studies of the Role of Factor VII in Hemostasis

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The effect of both congenital and acquired factor VII deficiency on the cuticle bleeding time (CBT) was evaluated in dogs. The CBT has been previously documented to be a sensitive indicator of factor VIII:C deficiency in hemophilic dogs. Serial CBT determinations were made on normal dogs treated with high-dose warfarin. At 48 hours post-treatment, the CBT was normal, although the factor VII level was <1%, whereas the levels of factors II, IX, and X were 44%, 25%, and 17%, respectively. At 120 hours the CBT became abnormal when all vitamin K-dependent clotting factors had dropped to <18%. Administration of a plasma concentrate of factors II, IX, and X corrected the CBT, despite the factor VII level remaining at <1%. Similar studies in a congenitally factor VII-deficient dog (factor VII <2%) confirmed that this deficiency state was not associated with an abnormality of the CBT. Administration of heparin to both normal and factor VII-deficient animals was associated with prolongation of the CBT, but the heparin dose required in the normal animals was substantially higher than in the factor VII-deficient animals. These data do not suggest that factor VII/VIIa has an exclusive role in generating factor Xa, either directly or indirectly, by way of factor IXa generation, in vivo. However, the increase in heparin sensitivity of the factor VII-deficient animals does suggest that factor VII/VIIa may, in some circumstances, present a significant alternative pathway of factor X activation, although the activation pathway involved cannot be determined from the studies performed.

ALTHOUGH SIGNIFICANT ADVANCES have been made during the past two to three decades in the understanding of the mechanisms of coagulation, the precise process by which the sequence of enzymatic events leading to thrombin generation is initiated remains controversial. The use of simple coagulation assays suggested the presence of two independent pathways capable of triggering this event. Patients with hemophilia A were found to have normal prothrombin times, whereas those with severe factor VII deficiency had normal clotting times in assays initiated by contact activation. These observations lead to the classic description of the extrinsic and intrinsic pathways that persists to this day. This proposes two alternative mechanisms for the activation of factor X to factor Xa. In the extrinsic pathway, this is mediated by factor VII/VIIa in association with tissue thromboplastin and calcium. In the intrinsic pathway, activation is achieved through factor IXa in the presence of factor VIII:C, phospholipid, and calcium. This concept presents several dilemmas if the known consequences of discrete coagulation factor deficiencies are considered. It is known that deficiencies of factors VIII:C and IX are associated with a severe bleeding diathesis. This would appear to be incompatible with the classic description of the extrinsic pathway, given that it should provide an adequate alternative means of factor Xa generation. Similarly, factor IXa is generated after a sequence of events that follow the contact activation of factor XII and the subsequent generation of factor Xa and factor IXa in sequence. Yet deficiencies of factor XII and Fletcher and Fitzgerald factors are not associated with a bleeding diathesis. Moreover, the bleeding diathesis in factor XI deficiency is only moderate relative to that seen with equivalent deficiencies of factors IX and VIII. With these contradictions in mind, the description by Østerud and Rapaport of the potential of factor VII/VIIa to activate factor IX in the presence of tissue thromboplastin in vitro was of significant interest. These observations, subsequently confirmed by further in vitro studies, provided an alternative pathway for the activation of factor X that could account for the severe bleeding tendency noted in deficiencies of factors VIII:C and IX. However, clinical observations are not completely in accord with an exclusively pivotal role for factor VII in vivo. Whereas some patients with severe factor VII deficiency do have a severe bleeding tendency, others with similar functional levels of factor VII do not. Similarly, although factor XI deficiency is not usually a severe disorder, such patients do bleed abnormally after surgery and, in some cases, it can be severe. The availability of a simple and reproducible animal model sensitive to a discrete deficiency of factor VIII:C enabled us to address some of these questions experimentally in vivo. It could be expected that if the activation of factor X by factor VII(a) in vivo occurs predominantly through factor IX activation, the CBT should be prolonged in factor VII-deficient animals. Both congenital and acquired factor VII-deficient animals were studied.

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MATERIALS AND METHODS

Animals

Three normal male mongrel dogs weighing between 10 and 12 kg were obtained from the Queen’s University Animal Facility. Each was screened for coagulation abnormalities (see later) before being used in the studies described. A purebred female beagle with severe congenital factor VII deficiency (factor VII <2%) was purchased from Marshall Research Animals, Inc, Northrose, NY. All animals were maintained on water ad libitum and regular dry dog chow (Ralston-Purina, St Louis).

Cuticle Bleeding Time Determination

The cuticle bleeding time (CBT) was performed as previously described on lightly anesthetized dogs (Bio-Tal [MTC Pharmaceuticals, Mississauga, Canada] 5%, 18 mg/kg body weight).9 The period of observation after cutical injury was 12 minutes. Cuticles bleeding were obtained from the Queen’s University Animal Facility. Each animal was screened for coagulation abnormalities before being used in the studies described. A purebred female beagle with severe congenital factor VII deficiency (factor VII <2%) was purchased from Marshall Research Animals, Inc, Northrose, NY. All animals were maintained on water ad libitum and regular dry dog chow (Ralston-Purina, St Louis).

Studies on Anticoagulated Animals

Animals were anticoagulated with warfarin sodium (Coumadin, Endo Laboratories Inc, Garden City, NY) 0.76 mg/kg intramuscularly (IM) as a loading dose. This was supplemented as necessary intravenously (IV), based on the prothrombin time, which was performed twice daily. The warfarin effect was reversed either by the IV administration of vitamin K1 (Aquamephyton, Merck Sharp & Dohme, Pointe Clare, Dorval, Quebec) or human prothrombin complex concentrate (PCC; Konyne-Cutter Labs, Inc, Berkeley, Calif). The batch of PCC used had previously been determined to be relatively factor VII-deficient.

Coagulation Assays

Blood for coagulation assays was anticoagulated with sodium citrate (3.8% wt/vol), 9 vol of blood to 1 vol of anticoagulant. Blood was obtained by venipuncture using a 21-gauge butterfly needle (Abbot Ireland Ltd, Sligo, Ireland) and a two-syringe technique.

The prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured as previously described.9 Factor IX was assayed using a one-stage APTT technique and a normal canine pool plasma (obtained from ten healthy dogs) as a reference standard. Due to the unavailability of factor IX-deficient canine plasma, the substrate plasma used was human factor IX-deficient plasma (George King Biomed, Inc, Overland Park, Kan). Factors II, VII, and X were assayed by one-stage PT technique, and the same normal canine pool was used as a reference standard. In the case of factors II and X, the substrate-deficient plasmas used were also human. In the factor VII assays, either human or canine factor VII-deficient plasma was used. The thromboplastins used were either canine, prepared according to the method of Thomson as a saline extract of canine brain,18 or rabbit brain (Simplastin, General Diagnostics, Morris Plains, NJ). The latter was diluted 1:10 when canine rather than human factor VII-deficient plasma was used as the substrate. When undiluted Simplastin was used, the shorter clotting times obtained with the totally canine system reduced the sensitivity of the assay. Using this modification, there was close agreement in the assay of factor VII at all levels irrespective of whether human or canine factor VII-deficient plasma was used as a substrate, or whether rabbit or canine brain was used as a source of thromboplastin. This is in agreement with published studies in normal and VII-deficient dogs.18 Factor VII assays were also performed using the coupled amidolytic assay described by Seligsohn and co-workers,12 using the chromogenic substrate Bz-Gly-Glu-Arg-p-nitroanilide (S-2222) (Ortho Diagnostics, Raritan, NJ).

All animals were screened by APTT, PT, and CBT before inclusion in the studies. Factor assays were performed only when an abnormality was found in one of these screening tests. Heparin assays were performed by protamine sulfate (Eli Lilly Canada Inc, Toronto) titration.13

Platelet aggregations were performed using a Payton Aggregometer (Payton Assoc, Toronto) and platelet-rich plasma (PRP) obtained from animals before and after heparinization. Blood was anticoagulated with sodium citrate (3.8% wt/vol, 9 vol of blood to 1 vol of anticoagulant) and PRP obtained by centrifugation at 190 g for 15 minutes. Aggregations were performed with sodium arachidonate (Type-1, Sigma Chemical Co, St Louis), and the minimum concentration (final) to produce aggregation for pre- and post-heparin samples was determined. The platelet count was adjusted to 186 × 109/L with platelet-poor plasma (PPP) obtained from the same sample. Platelet counts were determined using a Coulter S-plus particle counter (Coulter Electronics Inc, Hialeah, Fla).

RESULTS

The Effect of Warfarin Therapy on CBT

A normal animal was treated with warfarin sodium at a loading dose of 0.76 mg/kg IM. CBTs were performed pre-treatment and at 48, 70, and 120 hours post-treatment. At the time of each CBT, blood was drawn and assays of factors II, VII, IX, and X performed by one-stage clotting assays. Factor VII was also measured by coupled amidolytic assay. The results obtained are shown in Fig 1. The pre-warfarin CBT was within the normal range established in our previous studies, 6.0 ± 3.7 minutes (SD).9 It remained within the normal range at 48 and 70 hours post-initiation of warfarin therapy despite the fact that the factor VII level was <1% on both occasions, measured either by clotting or amidolytic assays. At 120 hours the CBT prolonged to >12 minutes, but by this time the other vitamin K-dependent clotting factors had all fallen below 18%. At this stage, administration of a factor VII-depleted prothrombin complex concentrate at a dose calculated to elevate the factor IX level to 30%, corrected the CBT 30 minutes post-infusion. At this time the factor VII level remained <1%, but the levels of the other three clotting factors were corrected as shown. Similar results were obtained in two additional normal animals. Two studies were performed on each animal, making a total of six experiments. Administration of vitamin K1 IV also corrected the CBT within six hours, with an associated rapid correction of the clotting factor deficiencies (results not shown).

Determinations of the CBT in multiple experiments (n = 10) in the congenitally factor VII-deficient dog (factor VII 1.7% and 1.4% by clotting and amidolytic assay, respectively) were always within the normal range.
**Effects of Heparin Therapy on CBT in Normal and Factor VII-Deficient Animals**

Heparin was administered as a rapid bolus at various dosage levels to lightly anesthetized normal dogs. CBTs were performed before infusion and at 15, 30, and 60 minutes post-infusion. Blood was drawn before the determination of each CBT, and heparin assays performed by protamine sulfate neutralization titration. The results of a typical experiment are shown in Fig 2. Fifteen minutes after a bolus infusion of 5,000 units, the CBT was prolonged to >12 minutes and the heparin level was determined to be 4.77 U/mL. Fifteen minutes later, when the heparin level had fallen to 3.07 U/mL, the CBT had corrected and remained normal thereafter. Repeat studies in other normal animals demonstrated that the CBT was never prolonged when the plasma heparin level was <4.5 U/mL (n = 10). In contrast, administration of much smaller doses of heparin to the factor VII-deficient animal (n = 3) was associated with prolongation of the CBT when the heparin level was <2 U/mL (Fig 3). Similar results were found with warfarinized normal animals during the first 24 hours, when only the factor VII levels were significantly reduced (n = 2). Platelet aggregation studies and counts performed before and after heparinization were unchanged in both normal and factor VII-deficient animals, and therefore could not account for the differences noted in the CBT response to heparin.

**DISCUSSION**

We have previously demonstrated that the CBT is a sensitive indicator of factor VIII:C deficiency in congenitally factor VIII:C-deficient dogs. The CBT abnormality can be corrected by the administration of factor VIII:C concentrates, and the levels required closely approximate those known to represent the minimal requirements of this co-factor in human hemostasis. Because these animals have a discrete factor VIII:C deficiency, ie, they have normal factor VII levels, direct activation of factor X by factor VII(a) through the extrinsic pathway does not appear to provide an alternative pathway to correct the hemostatic abnormality assessed by this test. However, these observations would be compatible with the hypothesis that the preeminent activation pathway of factor X by factor VII(a) in vivo is through the activation of factor IX by factor VII(a). The results obtained in our studies with both congenital and acquired factor VII-deficient animals failed to support this hypothesis, as the predicted hemostatic defect was not observed. Hovig et al obtained similar results using a mesenteric capillary bleeding time model. In contrast to factors VIII- and IX-deficient animals, no abnormality was noted in factor VII-deficient dogs.

Seligsohn et al have demonstrated a discrepancy in factor VII levels in warfarinized patients between results obtained using an amidolytic assay as opposed to a clotting assay, the former giving higher values. They attributed this to the presence of partially carboxylated factor VII and that this was more effective in the amidolytic assay than the clotting assay due to differences in the incubation periods of the two assays, this being longer in the former. We considered the possibility that such qualitative abnormalities, although affecting the clotting assays, may provide sufficient activity in vivo to support the hemostatic response in the injured cuticle. However, it would appear that at very low levels of factor VII, ie, <2%, there is close agreement between the two assays in both warfarinized and congenitally factor VII-deficient animals.

The heparin studies were performed in an attempt to evaluate the possibility that factor VII/VIIa may
nonetheless provide an alternative pathway for factor X activation in vivo, either directly or indirectly, via factor IXa. Factor VII/VIIa has been shown to be relatively resistant to heparin/ATIII inhibition in contrast to the other serine proteases.\textsuperscript{16} Consequently, we speculated that if activation of factor X by factor VII/VIIa by either route presented a significant alternative in vivo, in this situation the absence of factor VII may be associated with augmentation of the anticoagulant effect of heparin due to the expected inhibition of factors IXa and XIa. Abnormalities of the CBT in the VII-deficient animals were associated with substantially lower plasma heparin levels than those seen in association with abnormal CBTs in the normal animals. These data suggest that factor VII(a) shares the important responsibility for the activation of factor X in vivo, either directly or indirectly, through the activation of factor IX, and in certain situations it may play the predominant role.

Our studies do not allow us to identify with certainty the alternative activation pathway(s) involved. However, on the basis of present knowledge, factor Xa is an obvious additional candidate. Although not usually severe, patients with severe factor XI deficiency do have a significant abnormality of hemostasis. This observation has also been made in dogs with severe factor XI deficiency.\textsuperscript{17} The severity of bleeding in either factor VII or factor IX deficiency may relate to the nature of the challenge to hemostasis. Generally speaking, in humans, factor VII deficiency would appear to be the more serious condition,\textsuperscript{7} although in dogs the opposite would appear to be the case.\textsuperscript{17,18} With this in mind, it is important to stress that care should be taken in extrapolating freely from our results, in dogs, to the human condition. It is possible that evolutionary influences have resulted in differing requirements for factor VII in the two mammalian species. Nonetheless, our observations suggest consideration of additional or alternative explanations for the observed heterogeneity of the clinical consequences of isolated factor VII deficiency and the relatively mild bleeding diathesis associated with factor XI deficiency. Combined deficiency of factors VII and XI may have more serious clinical consequences. We are not aware of any reports of such a congenital deficiency state or adverse reactions after the administration of vitamin K antagonists to factor XI-deficient patients. Such studies could be performed in XI-deficient animals, but unfortunately such animals are not available at the present time. We suggest that patients with significant bleeding associated with factor VII deficiency should be reevaluated as to the possible coexistence of an abnormality of the intrinsic pathway of factor IX activation, as this might explain why some patients with an apparently equivalent deficiency are more or less severely affected.\textsuperscript{7}

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