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GENE TARGETING IN HEMOSTASIS. FACTOR XI

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1. ABSTRACT

Factor XI (FXI) is the zymogen of a plasma serine protease (FXIa) that contributes to hemostasis by activating factor IX (FIX). This reaction appears to be important for sustaining thrombin production after initial fibrin formation, to consolidate and protect fibrin clots from degradation by fibrinolysis. Humans with congenital FXI deficiency have a variable propensity to bleed after trauma or surgery, but do not experience the "spontaneous" hemorrhage in joints and soft tissue characteristic of hemophilia (FVIII or FIX deficiency). Mice homozygous for a disruption of the FXI gene *($FXI^{-/-}$)* have prolonged activated partial thromboplastin times and no detectable plasma FXI activity. Like their human counterparts, $FXI^{-/-}$ animals are generally healthy, reproduce normally, and do not develop spontaneous hemorrhage. In tail bleeding time assays, $FXI^{-/-}$ animals may have slightly prolonged bleeding compared to $FXI^{+/+}$ and $FXI^{+/-}$ animals, however, a consistent hemostatic deficit has not been identified. More impressive results are obtained when $FXI^{-/-}$ mice are crossed with protein C deficient mice. Severe FXI deficiency partially ameliorates the devastating hypercoagulable state associated with severe protein C deficiency, indicating that FXI plays a role in certain thrombotic conditions.

2. INTRODUCTION

Factor (F) XI is the zymogen of the plasma serine protease FXIa, which contributes to normal hemostasis by activating FIX in a calcium-dependent manner (1-3). Once thought to be a component of a mechanism for initiation of fibrin formation, data collected over the past decade strongly suggest that FXI is required for sustained thrombin production after clot formation. In current models of hemostasis (*e.g.*, figure 1) initiation of coagulation occurs when FVII/VIIa in plasma is exposed to tissue factor (TF) at a site of blood vessel injury (4-7). The FVIIa/TF complex activates small amounts of FX and FIX, with subsequent generation of thrombin. Thrombin generated early in the process starts fibrin formation and activates FV and FVIII. However, FVIIa/TF activity is temporally limited by the protease inhibitor tissue factor pathway inhibitor (TFPI), which binds to FXa and neutralizes FVIIa/TF. Further production of FXa and thrombin to complete coagulation requires FIXa and FVIIIa. In this system, activation of FIX by FXIa sustains coagulation after inhibition of FVIIa/TF by TFPI (8,9). Thus, in situations where hemostasis is severely challenged, the FIXa produced by FVIIa/TF may be insufficient to complete coagulation and must be supplemented through FXIa. This model fits well with the bleeding

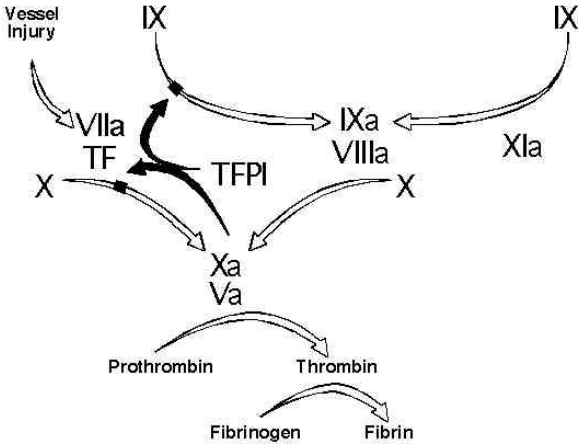

Figure 1. A model of plasma coagulation protease reactions in hemostasis. Fibrin clot formation is initiated by exposure of FVIIa in plasma to tissue factor (TF) at a site of blood vessel injury. The FVIIa/TF complex activates some FX and FIX to initiate fibrin formation through the production of thrombin. The tissue factor pathway inhibitor (TFPI) inhibits FVIIa/TF in a FXa dependent manner, preventing further activation of FX and FIX through this complex (black arrows and boxes). FIXa, in the presence of FVIIIa, continues FX activation to sustain thrombin production. In certain situations (severe hemostatic challenges or wounds in areas of high fibrinolytic activity), additional FIX is activated by FXIa. Roman numerals indicate the plasma clotting factors. The small "a" after the Roman numeral indicates the activated form of the clotting factor.

abnormalities seen in FXI deficiency in humans, which typically occur after trauma or surgery, or in tissues with high fibrinolytic activity such as the oropharynx and urinary tract (10,11).

Sustained thrombin production through FXIa appears to be important for activities beyond conversion of fibrinogen to fibrin. von dem Borne and colleagues demonstrated that trace amounts of FXIa render clots relatively resistant to fibrinolysis *in vitro* (12). This effect may be mediated by a thrombin-activated metaloproteinase called TAFI (thrombin-activatable fibrinolysis inhibitor) which modifies fibrin leading to reduced plasmin generation and activity (13,14). The anti-fibrinolytic activity of FXI was demonstrated *in vivo* by Minnema *et al*., who showed that inhibiting FXI with an antibody renders clots more susceptible to fibrinolytic therapy in a rabbit jugular vein thrombosis model (15). The findings suggest that FXI could contribute to thrombotic disorders by countering fibrinolysis. To assist us in our investigation of FXI function in normal hemostasis and thrombosis, we prepared mice with a targeted disruption of the FXI gene.

3. THE FXI MOLECULE

3.1. The structures of human and murine FXI

Human FXI is a 160 kDa plasma protein that circulates in a non-covalent complex with the glycoprotein high molecular weight kininogen (16,17). FXI is a dimer of two identical 80 kDa polypeptides connected by a single disulfide bond (16,18,19). A diagram of the primary and disulfide bond structures of human FXI monomer is shown in figure 2. As with other coagulation and fibrinolytic enzymes, the C-terminal portion of the polypeptide is a trypsin-like serine protease domain (5,18). The N-terminal region is comprised of four disulfide bond-constrained 90-91 amino acid repeats called apple domains (designated A1-A4) (18,19). In contrast to other coagulation proteases, FXI lacks an N-terminal calcium binding γ-carboxyglutamate (Gla)-domain (5,18,20,21). Gla domains are critical for protease interactions with phospholipids (20,21), and the absence of this domain may explain why FXIa-mediated reactions are not influenced by phospholipid (1,2,22). While an analysis of disulfide bond structure for murine FXI is not available, the human and murine proteins are 78% identical in amino acid sequence and have cysteine residues in identical locations (23). Recombinant murine FXI, like its human counterpart, is a disulfide bond-linked dimer. The available data, therefore, suggest that the human and murine proteins are structurally similar.

The apple domains of the FXI heavy chain differ from subunits found in the vitamin K-dependent proteases of the coagulation cascade, or the enzymes of fibrinolysis (5,21). While apple domains have homology with the N-terminal "PAN domains" of plasminogen and hepatocyte growth factor (24), the plasma protease prekallikrein (PK) is the only other protein identified to date with apple domains (25,26). Indeed, FXI and PK share such a high degree of homology both in protein and gene structure, that they are undoubtedly the products of gene duplication (discussed below) (27,28). Using a variety of techniques, roles in protein-protein and protein-surface interactions have been assigned to the apple domains of human FXI (see the legend of figure 2). It is important to note that there are contradictory results in the literature, so some of these assignments should be considered tentative. An analysis of the functions of the apple domains is not available for murine FXI, however, the high degree of sequence homology with the human protein suggests that it has similar structure/function relationships.

3.2. Activation of FXI and properties of FXIa

Human FXI circulates in plasma at a concentration of ~5 μg/ml (30 nM), which corresponds to a specific activity of 200 units/mg protein in an activated partial thromboplastin time (aPTT) assay (1 unit is the amount of activity in one ml of normal human plasma) (16). When recombinant murine FXI is used to reconstitute human FXI-deficient plasma in this type of system, it has a specific activity of ~140 units/mg of protein, indicating it functions in a fairly similar manner to human FXI (23). In vitro, both human and murine FXI are activated to FXIa by a single proteolytic cleavage in each polypeptide (between Arg^{369} and Ile^{370} in humans and Arg^{371} and Val^{372} in mice, see figure 2), resulting in a 45-50 kDa "heavy chain" containing the apple domains, and a 35 kDa catalytic light chain (23). Human and murine FXI are activated by human thrombin and FXIIa in purified protein systems. In contrast to human FXI, the murine protein undergoes autoactivation

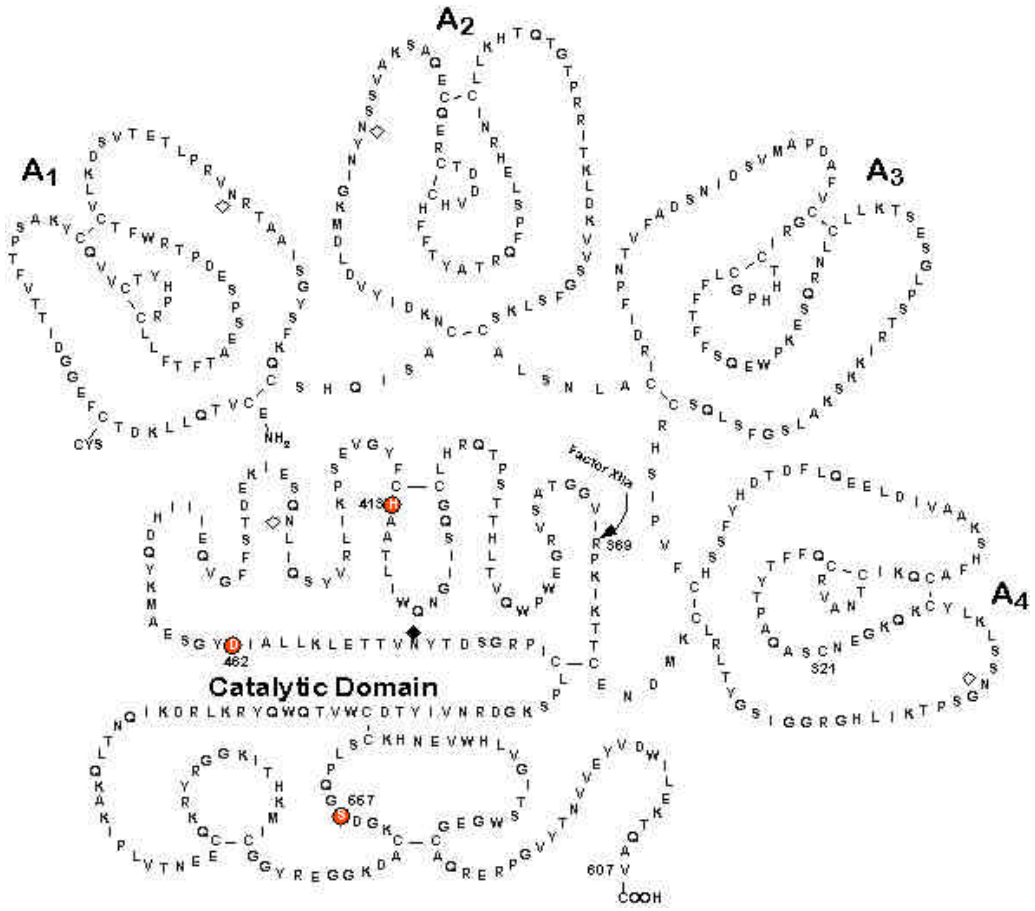

Figure 2. Amino acid sequence and disulfide bond structure of human FXI monomer. The four apple domains of the heavy chain are designated A1-A4. The free cysteine at position 321 in the A4 domain is involved in the interchain disulfide bond between the two polypeptides of the FXI homodimer. The Arg^{369}-Ile^{370} bond cleaved by FXIIa, thrombin or FXIa during FXI activation is designated by the curved arrow. The circled residues comprise the catalytic triad of the serine protease catalytic domain. The A1 domain appears to be involved in binding interactions with high molecular weight kininogen (55), prothrombin (34,56), and thrombin (57), the A3 domain in platelet binding (38,58), and the A4 domain in binding to FXIIa (59). The A3 domain (38,40) and A2 domains (60) have both been identified as binding sites for FIX. (After Fujikawa et al. 1986, and McMullen et al. 1991, Copyright, with permission from the American Chemical Society).

poorly in the presence of dextran sulfate or glycosaminoglycans (23,29). Murine FXIa activates human FIX with similar kinetic parameters (K_m = 0.15 µM, k_{cat} = 1.5/min) to the reaction mediated by human FXIa (K_m = 0.22 µM, k_{cat} = 3.5/min) (23).

3.3.FXI/XIa binding interactions with FIX and platelets

In the classic cascade or waterfall hypotheses of fibrin formation, FXI is activated by FXIIa during contact activation (30,31). However, the absence of excessive bleeding associated with severe FXII deficiency suggests this reaction may not be physiologically important (32). At the very least, the clinical data suggest that alternative mechanisms for FXI activation must exist. FXI activation by thrombin and autoactivation have been demonstrated in purified protein systems (8,33), and on the surface of activated platelets (34). Indeed, recent work by Baglia and co-workers support the premise that the platelet surface is a physiologic site for FXI activation, and that thrombin is superior to FXIIa as a FXI activator in this environment (35). As FIX also binds to activated platelets (36), it is likely that FIX activation by FXIa also occurs on the platelet surface (37).

We have studied the roles of the individual FXI apple domains in binding interactions with FIX and platelets using a panel of recombinant chimeric molecules in which individual apple domains of FXI are replaced with corresponding domains from PK (38,39). As PK does not interact well with FIX or platelets, the chimeric proteins have proven to be useful reagents for studying FXI structure/function relationships. The K_m for activation of FIX by FXI with a PK substitution in the A3 domain is ~20-fold greater than for activation by wild-type FXIa or

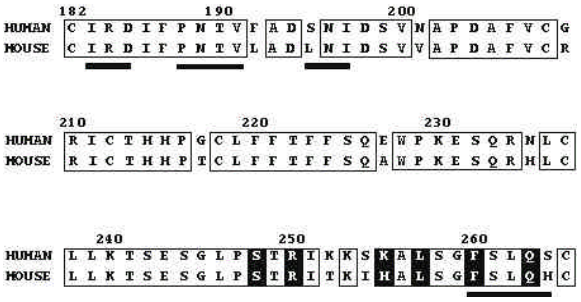

Figure 3. A comparison of the apple 3 domains of human and murine FXI. Amino acids 182 through 265 of the human and mouse FXI sequences are shown side-by-side. Amino acids that are identical in the two species are enclosed in the light gray boxes. Residues involved in the putative FIX binding site are underlined. Amino acids required for binding to activated platelets are contained in the dark gray boxes.

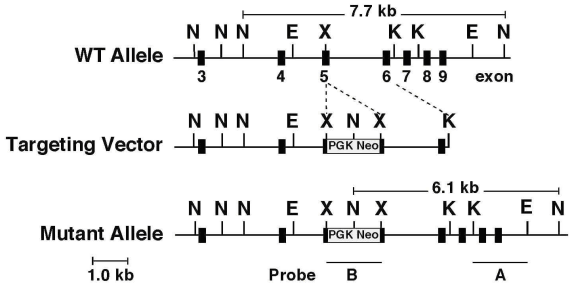

Figure 4. Targeting strategy for the disruption of the murine FXI gene. Schematic diagrams of exons 3 through 9 of the wild type murine *FXI* gene (top), targeting construct (middle), and the homologous recombinant (null) *FXI* gene (bottom). Restriction endonuclease sites: Nco I (N), Eco R1 (E), Xho I (X) and Kpn I (K). Location of the probes for Southern blot analysis are shown at the bottom of the figure.

chimeras with the PK A1, A2, or A4 domain (38). This strongly indicates that the A3 domain is required for normal binding to FIX. Subsequent mutagenesis studies identified two clusters of amino acids at the N- and C-terminal regions of A3 required for normal FIX activation (figure 3) (40). Comparison of the human and murine sequences demonstrates that the proposed FIX binding site is highly conserved between the species (23); a finding consistent with the kinetic studies described in section *3.2*. Using a similar strategy, amino acids required for binding of FXI to activated platelets were identified in the C-terminal region of A3 (figure 3) (39). Again, the residues critical for platelet binding in the human protein are conserved in the mouse. The conservation of sequence in these critical areas strongly implies that the biochemistry of murine and human FXI are similar.

4. TARGETED DISRUPTION OF THE MURINE FXI GENE

4.1. The FXI gene

In humans, the genes for FXI and PK have the same number of exons, identical intron/exon boundaries, and are both located on the same area of chromosome 4 (4q34-35) (27,28,41,42). Similarly, the genes for murine FXI and PK are in close proximity to each other on chromosome 8 (43). These findings clearly indicate that the two genes are derived from a common ancestral gene through a duplication event. In humans, FXI mRNA species of 2.4 and 4.4 kb have been identified by northern immunoblot in mRNA from liver, pancreas and kidney; and reverse transcription-PCR techniques have identified FXI mRNA in platelets and bone marrow (probably megakaryocytes) (23,44). In contrast, the 2.8 kb murine FXI mRNA has been identified only in liver (23). While the reasons for the difference in tissue specific expression are not clear, the findings suggest that FXI may serve somewhat different functions in the two species. The human FXI gene is comprised of 15 exons spread over ~ 25 kilobases (27), and exons 2 through 15 of the murine FXI gene have been cloned and partially sequenced (45). The intron/exons boundaries are identical in the two species.

4.2. Strategy for gene disruption

A 6.5 kilobase *Sal* I - *Kpn* I fragment encompassing exons three through six of the murine *FXI* gene was subcloned to create the targeting vector (figure 4). A cassette containing the neomycin phosphotransferase gene *(NEO)* with the phosphoglycerate kinase promoter and a bovine growth hormone polyadenylation site (provided by T. Ley, Washington University, St. Louis, MO), was cloned into a unique *Xho* I site in exon five. Exon five encodes the N-terminal half of the A2 domain (figure 2) (18,19,27), and a *FXI* message truncated at this point would lack most of the heavy chain and the entire catalytic light chain. This construct was transfected by electroporation into RW4 murine embryonic stem cells, and clones with homologous recombination of the targeting vector were identified by Southern blot (45). Properly targeted cell lines were injected into C57Bl/6 blastocysts, and injected blastocysts were implanted into pseudo-pregnant foster females using standard techniques. Germline transmission was achieved and pups heterozygous, and ultimately homozygous for the disrupted gene were generated.

5. THE PHENOTYPE ASSOCIATED WITH FXI GENE DISRUPTION

5.1. FXI deficient mice (45)

Mice heterozygous *($FXI^{+/-}$)* and homozygous *($FXI^{-/-}$)* for the targeted disruption appear and grow normally, and have a normal life span. No evidence of spontaneous bleeding has been noted in homozygous null mice either in the original mixed background or when the *$FXI^{-/-}$* genotype was backbread onto the C57/Bl6 background (D. Gailani, unpublished observation). The genotypes of progeny of parents heterozygous for the gene disruption are in the expected 1:2:1 ratio (wild type:

Table 1. Coagulation parameters for wild type and FXI null mice

		FXI genotype		
Assay	**PNP (control)**	+/+	+/-	-/-
aPTT (sec)	26 - 28	25 - 37	40 - 61	158 - >200
PT (sec)	8 - 10	8 - 12	10 - 12	10 - 12
FXI%	100*	54 - 64	17 - 20	<1

Ranges for aPTT and PT assays are given in seconds and represent the results of duplicate assays performed on the plasma of three animals of each genotype. Ranges for the FXI assay are given in % of normal control and represent averages of duplicate results on two pools of plasma; each pool containing the plasma of three individuals. Ranges for pooled normal murine (PNP) plasma (Swiss Webster strain) represent the results of assays performed in triplicate. Symbols designating FXI genotype: (+) wild type FXI allele, (–) null FXI allele. Abbreviations: PTT- activated partial thromboplastin time, PT- prothrombin time, FXI% - FXI concentration as a percentage of the concentration in the PNP control. *The FXI% for the murine PNP was arbitrarily assigned a value of 100% for the purposes of constructing the control curve.

Table 2. Results of the tail transection bleeding time assay

Genotype	**Number of Animals**	**Mean Bleeding Time (min)**	**Median Bleeding Time (min)**	**Bleeding Time Range (min)**
+/+	14	2.55	1.75	1.33 - >10
+/–	23	3.11	2.17	1.17 - >10
–/–	12	4.27	2.75	1.42 - >10

Tails of 6-8 week old mice were amputated 5-10 mm above the tip using a circular template with a diameter of 2 mm. The bleeding tail was immediately immersed in 37oC normal saline and the time to cessation of bleeding determined. The tails of mice that bled for >10 minutes were cauterized with silver nitrate to prevent exsanguination. Symbols for FXI genotype: (+) wild type FXI allele, (–) null FXI allele.

heterozygous null: homozygous null) indicating that the FXI null allele is not associated with increased intrauterine death. Homozygous null mice are fertile, and homozygous null females have normal sized liters of viable pups. This indicates that severe FXI deficiency in the mother does not prevent pregnancies from being carried to term. This is consistent with observations of pregnancies in humans with severe FXI deficiency (46). Northern blot analysis of polyA-RNA demonstrates reduced FXI message in the livers of FXI+/– mice, and the absence of message in FXI–/– animals. These findings were confirmed by reverse transcription-PCR, indicating that the disrupted gene fails to make a stable message coding for any part of the FXI molecule in liver.

5.2. FXI deficiency and hemostasis in mice (45)

Blood was collected by inferior vena caval puncture into a 1/10th volume of 3.8% sodium citrate and plasma was prepared by centrifugation. Plasma was tested in a conventional aPTT assay and in a prothrombin time (PT) assay using a thromboplastin reagent containing human tissue factor. Results are shown in table I. Mice deficient in FXI, as with FXI deficient humans, have a marked prolongation of the aPTT and a normal PT, consistent with a defect in the intrinsic pathway. Using a celite elution method (47), it was determined that mice homozygous for the null allele have <1% of the plasma FXI concentration of the normal plasma control. Of note, animals homozygous for the wild type allele have only 54-64% of the activity of the control. The normal pool of plasma used as control for these experiments comes from the Swiss Webster line, thus, there may be some inter-strain differences in plasma FXI levels.

The effects of FXI deficiency on hemostasis were assessed by a modification of the tail transection bleeding time of Dejana and co-workers (48). Fifty, 6-8 week old progeny of matings of mice heterozygous for the FXI null allele were tested. Tails were transected 5-10 mm from the tip using a template that insured transections would have a similar cross-sectional area. The tail stump was placed into saline at 37°C, and time to cessation of bleeding was determined. Normal (FXI+/+) mice typically bleed for 1-2 minutes, although an occasional animal has prolonged bleeding (>10 min). Median bleeding time is probably more meaningful than mean bleeding time for this assay, as animals that bleed >10 minutes are treated with wound cautery to prevent exsanguination. FXI–/– animals have, at most, a slight propensity to bleed longer than FXI+/+ or FXI+/– animals (table 2). However, the range of bleeding times is wide for all groups, and a reproducible bleeding abnormality is not demonstrated with this technique. Similarly, a toe amputation assay failed to distinguish a difference in bleeding between FXI+/+ and FXI–/– animals (D. Gailani, unpublished observation)

5.3. Effects of FXI deficiency on the phenotype of protein C deficient mice (49)

It is apparent that FXI deficiency is not associated with an easily induced hemorrhagic phenotype in mice on a mixed or C57Bl/6 background. An alternative strategy for identifying a phenotype in these animals is to examine effects of FXI deficiency on thrombosis. As FXI is hypothesized to be part of a mechanism that counters fibrinolysis, FXI deficiency may provide some protection from thrombosis. Therefore, crossing FXI deficient mice with animals carrying genetic predispositions for thrombotic disorders may be informative. Protein C (PC) is a plasma protease that down-regulates hemostasis by degrading the critical coagulation proteins FVa and FVIIIa (50). Mice homozygous for a null deletion of the *PC* gene die *in utero* or within a few days of birth from a severe disseminated coagulopathy that is similar to the syndrome of *purpura fulminans* associated with severe PC deficiency in humans (51,52). $FXI^{-/-}$ mice were crossed with $PC^{+/-}$ animals to generate $FXI^{+/-}/PC^{+/-}$ animals on the C57Bl/6 background. These animals were then bred in an attempt to generate double homozygous null animals (genotype $FXI^{-/-}/PC^{-/-}$).

In timed mating experiments, day 17.5 $FXI^{-/-}/PC^{-/-}$ embryos lacked the diffuse hemorrhage and fibrin deposition in brain, kidney, atria, and other soft tissues seen in day 17.5 $FXI^{+/+}/ PC^{-/-}$ animals. It should be noted, however that the $FXI^{-/-}/PC^{-/-}$ mice were not entirely normal, with most having opacifications in the liver, and one-quarter having some bleeding in the head or bladder. To date, 15 $FXI^{-/-}/PC^{-/-}$ have survived the perinatal period, with life spans of 13-94 days (F.J. Castellino, personal communication). The majority of these animals are sedentary and growth-retarded. At the time of natural death (or sacrifice) these animals have widespread heavy fibrin deposition associated with hemorrhage and fibrosis. Several animals had enlarged lymph nodes and large amounts of lymphatic fluid in the thorax. While it is clear that FXI deficiency cannot totally prevent the consequences of severe PC deficiency, this study provides the first clear indication that the natural progression of a severe thrombotic disorder can be altered by blocking the intrinsic pathway. In addition it presents an opportunity to examine the consequences of PC deficiency in adult animal.

6. PERSPECTIVE

The highly variable bleeding disorder accompanying congenital FXI deficiency in humans has presented a challenge for clinicians and basic researchers in the field of hemostasis. There is a growing consensus that FXI plays a role in consolidating and protecting fibrin clots after clot formation initiated through a FVIIa/TF mediated process. This is in stark contrast to earlier models that proposed a key role for FXI in initiation of fibrin formation through the intrinsic pathway. To aid us in investigating the importance of FXI to hemostasis, FXI-deficient mice were prepared by the standard technique of homologous recombination in embryonic stem cells. As expected, FXI-deficient mice are viable and fertile and, like humans with severe FXI deficiency, have marked prolongations of the aPTT. The FXI-deficient animals did not demonstrate a significant bleeding abnormality using techniques that easily demonstrate a hemostatic defect in mice with FVIII deficiency. In this respect, they are similar to some humans with severe FXI deficiency, who do not bleed despite trauma or surgery.

Recently, clinical studies in humans have suggested a role for FXI in thrombotic disorders. Meijers and colleagues presented data indicating that individuals with plasma FXI levels above the 90th percentile have a 2.2-fold increased risk for venous thrombosis compared to the general population (53). This risk appears to be independent of common inherited risk factors such as the FV Leiden and prothrombin gene G20210A polymorphisms. Along similar lines, Minnema and coworkers have observed evidence of increases in FXI activation in some patients experiencing acute coronary syndromes (myocardial infarction and unstable angina) but not stable angina (54). These observations are very intriguing in light of the proposed role of FXI as a counter-balance to fibrinolysis, and suggest that FXI may contribute to human pathologic conditions. The demonstration that FXI deficiency partially ameliorates the severe thrombotic phenotype of PC deficiency in mice further supports this premise (49). The FXI deficient mouse line, therefore, is a valuable tool for assessing the contributions of FXI to different thrombotic processes. To this end, FXI-deficient mice are currently being crossed with plasminogen deficient, ApoE-deficient, FV Leiden expressing, and plasminogen activator inhibitor-1 over-expressing mice.

7. ACKNOWLEDGMENTS

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