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Evidence for factor IX-independent roles for factor XIa in blood coagulation

Anton Matafonov^{*,†}, Qiufang Cheng^{*}, Yipeng Geng^{*}, Ingrid M. Verhamme^{*}, Obi Umunakwe^{*}, Erik I. Tucker[‡], Mao-fu Sun[‡], Vladimir Serebrov[§], Andras Gruber[‡], and David Gailani^{*,¶}

^{*}Department of Pathology, Microbiology, and Immunology, Vanderbilt University, Nashville, TN

[†]Department of Bioengineering and Organic Chemistry, Tomsk Polytechnic University, Tomsk, Russia

[‡]Department of Biomedical Engineering, Oregon Health and Science University, Portland, OR

[§]Department of Biochemistry and Molecular Biology, Siberian State Medical University, Tomsk, Russia

[¶]Department of Medicine, Vanderbilt University, Nashville, TN

Abstract

Background—Factor (f) XIa is traditionally assigned a role in fIX activation during coagulation. However, recent evidence suggests this protease may have additional plasma substrates.

Objective—To determine if fXIa promotes thrombin generation and coagulation in plasma in the absence of fIX, and to determine if fXI deficiency produces an antithrombotic effect in mice independent of fIX.

Methods—FXIa, fXIa variants, and anti-fXIa antibodies were tested for their effects on plasma coagulation and thrombin generation in the absence of fIX, and for their effects on activation of purified coagulation factors. Mice with combined fIX and fXI deficiency were compared to mice lacking either fIX or fXI in an arterial thrombosis model.

Results—In fIX-deficient plasma, fXIa induced thrombin generation and anti-fXIa antibodies prolonged clotting times. This process involved fXIa-mediated conversion of fX and fV to their active forms. Activation of fV by fXIa required the A3 domain on the fXIa heavy chain, while activation of fX did not. FX activation by fXIa, unlike fIX activation, was not a calcium-dependent process. Mice lacking both fIX and fXI were more resistance to ferric chloride-induced carotid artery occlusion than fXI-deficient or fIX-deficient mice.

Conclusion—In addition to its predominant role as an activator of fIX, fXIa may contribute to coagulation by activating fX and fV. As the latter reactions do not require calcium, they may make important contributions to in vitro clotting assays triggered by contact activation. The reactions may be relevant to fXIa's roles in hemostasis and in promoting thrombosis.

Address correspondence to: David Gailani, MD Division of Hematology/Oncology Vanderbilt University 777 Preston Research Building 2220 Pierce Ave. Nashville, TN 37232-6305 Phone # (615) 936-1505 FAX # (615) 936-3853 dave.gailani@vanderbilt.edu.

AUTHORSHIP A.M. conducted studies of plasma coagulation, fXIa cleavage of clotting factors, and thrombin generation, and wrote the original draft of the manuscript. Q.C. performed mouse thrombosis studies. Y.G. prepared and tested recombinant fXIa variants. I.M.V. Designed experiments on fX and fV activation and interpreted results. O.U. conducted studies on fX activation by fXIa. E.I.T. generated and characterizing antibodies against the fXIa A3 and catalytic domains. M-f.S. prepared recombinant proteins and identified binding epitopes of monoclonal IgGs. V.S., and A.G. contributed to overall study design and interpretation of data. D.G. was responsible for oversight of the project and preparation of the final manuscript.

Keywords

Factor XI; factor XIa; factor IX; factor X; factor V

INTRODUCTION

In the cascade/waterfall hypotheses of plasma coagulation [1–3], thrombin generation is the result of a series of proteolytic reactions initiated by conversion of factor (f) XII to the protease fXIIa. In the partial thromboplastin time (PTT) assay used in clinical practice, fXIIa forms when blood comes into contact with a charged surface through a process called *contact activation* [4,5]. FXIIa activates fXI to fXIa, which in turn converts fIX to fIXa β when plasma is recalcified. This set of reactions, referred to as the *intrinsic pathway*, while required for clot formation in the PTT assay, likely serves a minor role in hemostasis. FXII deficiency is not associated with abnormal bleeding [4,5], while fXI deficiency causes relatively mild bleeding compared to fIX deficiency [4–6]. However, there is mounting evidence that fXIa and fXIIa contribute to thrombosis [7–9], suggesting that reactions similar to those in the intrinsic pathway are involved in pathologic coagulation.

FXIa is usually assigned a single function in coagulation, activation of fIX [3,4]. Recently, Whelihan *et al.* observed that citrate-anticoagulated plasma supplemented with fIXa β clots slowly after recalcification compared to the same plasma after recalcification in the PTT assay [10]. They concluded that fXIa-mediated fIX activation alone cannot account for the rate of clot formation in the PTT, and showed that fXIa cleaves fVIII and fV to active forms. Here, we show that fXIa induces thrombin generation in fIX-deficient plasma by activating fV and fX. In a mouse arterial thrombosis model, combined fIX and fXI deficiency produced a greater antithrombotic effect than deficiency of either protein alone, consistent with the hypothesis that fXIa interacts with substrates other than fIX.

MATERIALS AND METHODS

Reagents

FV was prepared as described [11]. Normal human plasma; Precision BioLogic. FIX- or fV-deficient plasma; George King. FIX-depleted plasma and PTT-A reagent; Diagnostica Stago. FXIIa, fibrinogen and corn trypsin inhibitor (CTI); Enzyme Research. FVa, fIXa β , fX, fXa, fXIa, prothrombin; Haematologic Technologies. Aprotinin, hirudin; Sigma. Apixaban; ChemScene. Phosphatidylcholine-phosphatidylserine (PC-PS) vesicles; Avanti Polar Lipids. S-2366 (L-pyro-Glu-L-Pro-L-Arg-*p*-nitroanilide), S-2765 (Z-D-Arg-Gly-Arg-*p*-nitroanilide), and S-2238 (H-D-Phe-L-Pip-L-Arg-*p*-nitroanilide); DiaPharma. Z-Gly-Gly-Arg-AMC (Bachem).

Recombinant proteins

Wild type fXI (fXI^{WT}), fXI with the prekallikrein (PK) A3 domain (fXI/PKA3), and fXIa catalytic domain (fXIa^{CD}) were prepared as described [12]. fXI^{WT} and fXI/PKA3 were activated by incubation in 50 mM Tris-HCl pH 7.4, 100 mM NaCl (TBS) with fXIIa.

Monoclonal antibodies

Anti-fIX IgG SB249417 (from John Toomey, GlaxoSmithKline) [13] is designated Anti-IX^{Gla} for this study. Anti-fXI IgG aXIMab (O1A6) [14] is designated Anti-XI^{A3}. IgG Anti-XI^{AS} was raised by immunizing fXI-deficient Balb-C mice with fXIa catalytic domain.

Clotting assays

FIX-deficient plasma was incubated for 30 min at 4°C with vehicle or 300 nM Anti-IX^{Gla}, Anti-XI^{A3}, or Anti-XI^{AS}. Plasma (35 µl) and PTT-A reagent (35 µl) were mixed and incubated for 5 min at 37 °C. CaCl₂ (35 µL, 25 mM) was added and time to clot formation was measured on an ST-4 Analyzer (Diagnostica Stago). For fXIa initiated clotting, plasma was supplemented with 5 µM PC/PS vesicles. FXIa in TBS with 0.1% BSA (TBSA) was incubated for 30 min at 4°C with vehicle or 300 nM IgG (Anti-IX^{Gla}, Anti-XI^{A3}, or Anti-XI^{AS}). FIX-deficient human plasma, or plasma from mice with combined deficiencies of fIX, fXI and fXII (35 µL) were incubated with fXIa (35 µL) for 5 min at 37°C. Clotting was initiated with 25 mM CaCl₂ (35 µL).

Cleavage of fV, fIX, fX, and prothrombin

FV (1 µM), fX (500 nM), or prothrombin (500 nM) was incubated in TBS/0.1% PEG 8000 ± 1.2 mM CaCl₂ and fXIa (50 nM for fV and prothrombin, 125 nM for fX) at 37°C. Aliquots were removed at various times into non-reducing sample buffer. Prothrombin, fV, fIX, and fX (500 nM) were incubated with 50 nM fXIIa in TBS/0.1% PEG 8000 for 60 min at 37°C. Reactions were size-fractionated on 4–20% gradient polyacrylamide-SDS gels, stained with GelCode Blue, and imaged on an Odyssey infrared imager (LI-COR Biosciences) [12].

FVa activity

FV (30 nM) was incubated with fXIa (15 nM) in TBS with 0.1% PEG 8000 and 1.2 mM CaCl₂ at 37°C. 35 µl aliquots were supplemented with aprotinin and mixed with 35 µl fIX deficient plasma containing PC:PS vesicles (5 µM). FXa (1.25 nM) and CaCl₂ (6.7 mM) were added and time to clot formation determined. Values were converted to nM fVa activity by comparison to a standard curve prepared with fVa.

FXa activity

FX (150 nM) was incubated with 15 nM fXIa at 37°C in the absence or presence of 1.2 mM Ca²⁺, 1.0 mM Mg²⁺ or 10 µM Zn²⁺. Reactions were stopped with aprotinin (10 µM), and fXa cleavage of S-2765 was measured by following ΔOD₄₀₅ nm on a microplate reader. Values were converted to pM fXa by comparison to a standard curve. In separate assays, FX (150 nM) was incubated with fXIa (15 nM) in TBS with 0.1% PEG 8000 at 37°C with 300 nM Anti-IX^{Gla} or Anti-XI^{A3}. Aliquots were supplemented with aprotinin (10 µM), and mixed with prothrombin (100 nM), fVa (1 nM), PC:PS vesicles (5 µM) and CaCl₂ (1.2 mM). Thrombin cleavage of S-2388 was measured by following ΔOD₄₀₅ nm. Values were converted to pM fXa generated using a standard curve.

FXa activity in plasma

Normal or fIX-deficient plasma (100 µl) with or without Apixaban (10 µM) was incubated with PTT-A reagent (50 µl) at 37°C. At various times, reactions were stopped with CTI (4 µM), aprotinin (10 µM) and hirudin (10 µM). FXa cleavage of S-2765 was determined by following ΔOD₄₀₅ nm. Values were converted to pM fXa by comparison to a fXa standard curve.

Thrombin activity

Prothrombin (500 nM) was incubated with fXIa (125 nM) in TBS with 1.2 mM CaCl₂ and 0.1% PEG 8000 at 37°C. Reactions were stopped with aprotinin (10 µM). Cleaved prothrombin (60 nM) was tested for its ability to hydrolyze S-2238 (500 µM), and to convert fibrinogen (5 µM) to fibrin as measured with the ST-4 Coagulation Analyzer.

Thrombin generation assay

Plasma was supplemented with 415 μ M Z-Gly-Gly-Arg-AMC, 5 μ M PC/PS vesicles, 4 μ M CTI or vehicle, and 300 nM Anti-XI^{A3}, 900 nM Anti-IX^{Gla} or vehicle. Supplemented plasma (40 μ l) was mixed with 10 μ l Tyrode buffer pH 7.4 containing fXIa (final concentration 2.5 to 15 nM) or fIXa β (11–900 pM). Ten microliters of 20 mM HEPES, pH 7.4, 100 mM CaCl₂, 6% BSA was added and fluorescence (excitation λ 390 nm, emission λ 460 nm) was monitored at 37°C on a Thromboscope® [14]. Each condition was tested three times in duplicate. Peak thrombin generation and endogenous thrombin potential (ETP) were determined (Thromboscope Analysis software, 3.0).

Arterial thrombosis

C57Bl/6 mice were used in the study. FIX-deficient (fIX^{-/-}) mice were supplied by Darrel Stafford (U. North Carolina-Chapel Hill) [15]. FXI-deficient mice (fXI^{-/-}) were described [16]. fIX^{-/-} and fXI^{-/-} mice were crossed to generate mice lacking both proteins (fIX^{-/-}/fXI^{-/-}). Mice (20–25 gm) were anesthetized with pentobarbital (50 mg/kg IP). The right common carotid artery was exposed and fitted with a flow probe (Model 0.5 VB, Transonic System). Thrombus formation was induced by applying two 1 $\times$ 1.5 mm filter papers saturated with FeCl₃ (2.5 to 15%) to opposite sides of the artery for 3 min, followed by rinsing with normal saline [16,17]. Flow was monitored for 30 min. Results were compared with χ^2 -test.

RESULTS

Anti-fIX and anti-fXI antibodies in clotting assays

Three antibodies were used to study fXIa activity in plasma. Anti-IX^{Gla} is a potent inhibitor of fIX activation and fIXa β activity [13]. Anti-XI^{A3} blocks an exosite required for fIX activation on the fXIa A3 domain [12], and also inhibits fIX activation by fXIIa. Anti-XI^{AS} was raised against the fXIa catalytic domain. It appropriately recognizes fXI and the fXIa catalytic domain on western blots (Fig.1A), and inhibits fXIa cleavage of S2366 (Fig.1B) indicating it binds at the active site or alters its conformation. The PTT of normal plasma (45 $\pm$ 4 sec) was prolonged by Anti-XI^{A3} (180 $\pm$ 24 sec). Anti-XI^{AS} had a smaller effect (67 $\pm$ 4 sec), indicating it is not as potent as Anti-XI^{A3} at inhibiting fIX activation. Neither Anti-XI^{A3} (232 $\pm$ 16 sec) nor Anti-XI^{AS} (274 $\pm$ 21 sec) affected clotting in fXI-deficient plasma (259 $\pm$ 29 sec, Fig.1C), consistent with specificity for fXI/XIa.

The PTT of plasma from a fIX-deficient patient (Fig.2A, 106 $\pm$ 2 secs, antigen <0.1% normal), as expected, changed little with addition of Anti-IX^{Gla} (114 $\pm$ 12 sec), but was prolonged with Anti-XI^{A3} (154 $\pm$ 3 sec) and Anti-XI^{AS} (199 $\pm$ 2 sec) (Fig.2A). In contrast to its effect on normal plasma, Anti-XI^{AS} had a larger effect than Anti-XI^{A3} in fIX-deficient plasma. Similar results were obtained with fIX-depleted plasma (vehicle 142 $\pm$ 2, Anti-IX^{Gla} 156 $\pm$ 9, Anti-XI^{A3} 248 $\pm$ 10, Anti-XI^{AS} 379 $\pm$ 36 sec, Fig.2B).

Adding fXIa (3 nM) directly to fIX-depleted plasma induced clotting in 261 $\pm$ 8 sec. Anti-XI^{A3} (432 $\pm$ 26 sec) and Anti-XI^{AS} (>600, Fig.2C) again, prolonged time to clot formation (Figs. 2A and 2B), indicating they influence clotting by inhibiting fXIa, despite the absence of fIX. Clotting times in fXIa-initiated assays are longer than in PTT assays, because the PTT reagent induces generation of higher fXIa concentrations.

While a trace of fIX in fIX-deficient plasma could facilitate fXIa-initiated coagulation, it would not account for the large effects of the anti-fXI antibodies. We tested the ability of human fXIa to shorten the clotting time of plasma from mice deficient in all three intrinsic pathway proteases (factors IX, XI and XII, Fig.2D). The gene disruptions in these animals

prevent fIX, fXI and fXII synthesis [15,16]. This plasma was used because clotting times of plasma from mice lacking only one intrinsic protease are relatively short compared to comparable human plasmas, making them unsuitable for the assay. The results (Vehicle 219 ± 3 sec, 30 nM fXIa 179 ± 3 sec) support the premise that fXIa can promote clotting in a fIX-independent manner, although the effect is modest, perhaps due to the relatively short baseline clotting time of the plasma.

FXIa cleavage of fV

FV and fX are involved in the step downstream of fIX in the coagulation cascade. Whelihan *et al.* showed that fXIa cleaves fV, generating fVa activity [10]. The cleavage pattern is more complex than for fVa generated by thrombin, but the light and heavy chains and the B domain of fVa are evident (Fig.3A). Interestingly, the reaction proceeds faster without Ca^{2+} , suggesting that fXIa activation of fVa in the PTT assay would occur primarily during the contact phase before recalcification. FXIa with the A3 domain replaced with the PK A3 domain (fXIa/PKA3) cleaves the tripeptide S2366 similarly to fXIa^{WT}, but has a significant defect in fIX activation [12]. In reactions with fXIa/PKA3, the fV band disappeared at half the rate of reactions with fXIa^{WT} (Fig.3B and 3C), with slower fVa light chain (Fig.3D) and B-domain (Fig.3E) accumulation. Similar results were obtained with fXIa catalytic domain (fXIa^{CD}), which also lacks A3 (Fig.3B–3E). Anti-XI^{A3}, which binds to A3, reduced fVa light chain generation by fXIa ~50% (right panel Fig.3B), consistent with a role for the A3 domain in fV activation.

Addition of 30 nM standard fVa (generated with thrombin) induced clotting in recalcified normal plasma (586 ± 173 sec) but not fIX-deficient plasma (>900 sec) (Fig.3F). fVa requires fXa to produce thrombin, and the result could be explained by a higher fXa concentration in normal plasma than fIX-deficient plasma. Adding fXa to fIX-deficient plasma gave clotting times of 78 ± 3 sec, and addition of fXa and fVa shortened the clotting time further (45 ± 3 sec, Fig.3F). Based on these observations, we developed a fVa activity assay using fIX-deficient plasma supplemented with fXa. fV cleaved by fXIa had ~6% of the activity of an equivalent amount of standard fVa (Fig.3G), consistent with data from Whelihan *et al.* [10] showing a lower specific activity than standard fVa. The results also show that fV activation alone is insufficient to explain the capacity of fXIa to induce clotting in fIX-deficient plasma.

FX activation by fXIa

Activation of the homologs fIX and fX involves release of an activation peptide. FXIa cleaved fX to a product that migrated similarly to fXa (Fig.4A) on SDS-PAGE, although high fXIa concentrations were required to make this evident. At more physiologic concentrations, FX cleaved by fXIa shortened the clotting time of fIX-deficient plasma (216 ± 2 sec), while fX incubated without fXIa did not (Fig.4B). This effect is blocked by the fXIa inhibitor aprotinin (>450 sec)), but not appreciably by Anti-XI^{A3} (265 ± 3 sec, Fig.4B), indicating a minor role for the fXIa A3 domain in the reaction. Consistent with this, fX is activated similarly by fXIa^{WT}, fXIa/PKA3 or fXIa^{CD} (Fig.4C). Addition of divalent cations had little effect on fX activation by fXIa (Fig.4D), again suggesting that fX, unlike fIX, does not bind the fXIa A3 domain. FX cleaved by fXIa converted prothrombin to α -thrombin in the presence of fVa, and Ca^{2+} (Fig.4E). Purified plasma fX may contain fIX. Using a fIX-specific ELISA, we determined that 10 μg of fX contained ~0.3 ng of fIX (0.003%). Anti-IX^{Gla} prevented fX activation by fIXa β in the prothrombin activation assay (data not shown), but not affect fX activation by fXIa (Fig.4E), supporting the premise that fXIa, and not fIXa β , is activating fX.

These observations suggest that fXIa can convert fX to fXa during the PTT contact phase. This was demonstrated by incubating normal and fIX-deficient plasmas with PTT-A reagent without recalcification. After incubation, fXIIa, fXIa, and α -kallikrein were inhibited with CTI and aprotinin, and thrombin was inhibited with hirudin. Amidolytic activity toward the tripeptide S-2765 was noted in both plasmas that could be neutralized by the fXa-specific inhibitor apixaban (Fig.4F), consistent with fXa generation during contact activation.

Thrombin generation in the absence of fIX

Subpicomolar fXIa induces thrombin generation in normal plasma [14]. Nanomolar fXIa can induce thrombin generation in fIX-deficient plasma (Fig.5A); an effect that does not occur in the presence of apixaban or the absence of fV (Fig.5B). Thrombin is generated by as little as 10 pM fIXa β (Fig.5C), and this is blocked by Anti-IX^{Gla} (Fig.5D). Anti-IX^{Gla} was included in the studies in Fig.5A to account for possible fIX contamination. Cumulatively, the data support the hypothesis that fXIa is activating fV and fX in the absence of fIX.

Endogenous thrombin potential (1970 ± 10 vs. 1170 ± 15 nM.min) and peak thrombin generation (150 nM vs. 65 nM) were greater in fIX-deficient plasma supplemented with fXIa^{WT} than with fXIa/PKA3 (Fig. 5E). Recall that these proteases are equally effective fX activators, but fXIa^{WT} cleaves fV more effectively than fXIa/PKA3. The results in Fig. 5E are consistent with a scenario in which both proteases activate fX comparably, with fXIa/PKA3 having a reduced capacity to activate fV. In support of this, Anti-XI^{A3}, which interferes with fXIa activation of fV, but not fXIa activation of fX, reduced thrombin generation induced by fXIa^{WT} (1470 ± 10 vs. 650 ± 40 nM.min, respectively Fig.5F).

FIX-independent fXIa activity in vivo

Removing the tail tip of fIX^{-/-} mice with a scalpel typically leads to exsanguination [15], while bleeding in fXI^{-/-} mice is mild and comparable to wild type (WT) mice [16]. Double deficient fIX^{-/-}/fXI^{-/-} mice are viable, with a bleeding propensity similar to fIX^{-/-} mice. The double deficiency did not affect reproduction. Measurements of plasma fVIII, IX, XI and XII using one-stage clotting assays confirmed lack of activity due to specific gene disruptions, without significant differences in other factors (data not shown), indicating that loss of an intrinsic pathway factor does not cause large compensatory changes in other factors.

Despite the differences in bleeding propensity, fIX^{-/-} and fXI^{-/-} mice are comparably resistant to FeCl₃-induced carotid artery thrombosis (Fig.6) [17]. WT mice developed artery occlusion with 3.5% FeCl₃, while fIX^{-/-} and fXI^{-/-} mice are resistant to occlusion with 5% FeCl₃, and partially resistant at 7.5% ($p=0.001$ and 0.025 , respectively, compared to WT). If the thrombotic effect of fXIa is mediated exclusively through fIX activation, fIX^{-/-}/fXI^{-/-} and fIX^{-/-} mice should behave similarly in this assay. However, while fIX^{-/-} and fXI^{-/-} mice consistently occluded with 10% FeCl₃, some fIX^{-/-}/fXI^{-/-} mice did not occlude even at 12.5% FeCl₃ ($p=0.025$ and 0.06 compared to other genotypes for 10% and 12.5% FeCl₃, respectively). This is consistent with fXIa activating substrates in addition to fIX.

DISCUSSION

The catalytic domains of α -thrombin and the enzymes that contribute to its generation are homologs of the pancreatic protease trypsin [18]. While coagulation proteases demonstrate varying propensities to behave like trypsin (cleaving proteins after basic amino acids) when tested with pure substrates, they exhibit more restricted specificity in plasma. For these

proteases, substrate affinity and specificity are governed by exosite-interactions [19,20]. Exosites are substrate-binding sites that are distinct from the protease active site. FXIa contributes to thrombin generation primarily through Ca^{2+} -dependent activation of fIX. The gene for fXI is the result of a duplication of the PK gene [21]. For fXI, adaptation to a role as a fIX activator included changes to the parent PK sequence producing a high affinity fIX-binding exosite on the A3 domain [12,22]. While it is clear that fIX is the major plasma substrate for fXIa, there is evidence that it can act on a wider range of targets.

In the PTT assay, a charged substance added to plasma containing the Ca^{2+} chelator sodium citrate results in Ca^{2+} -independent contact activation of fXII, fXI and PK [4,5]. With recalcification, fXIa converts fIX to fIXa β , leading to clot formation. Welihan *et al.*, noting the limited capacity of fIXa β to activate fX without fVIIIa, and fXa to activate prothrombin without fVa, proposed that fVIIIa and fVa must form before recalcification (prior to fIX activation by fXIa) to explain the rate of clot formation in the PTT [10]. In support of this hypothesis, we showed that fXIa-mediated thrombin generation in fIX-deficient plasma depends on its capacity to activate fV in a reaction involving the fXIa A3 domain. This is consistent with work from Maas *et al.* showing that fV/fVa bind fXI through A3 [23].

In our study, thrombin generation in the absence of fIX required fXIa to activate fX, a result supported by recent data from Puy *et al.* [24]. The fIX gene arose from a duplication of the fX gene [25], and fIX and fX are structurally very similar. In the PTT, Ca^{2+} is required for proper folding of the fIX-Gla domain, which binds to the fXIa A3 domain [3,12,22]. FX activation by fXIa does not involve the A3 exosite, explaining why the reaction is not Ca^{2+} -dependent, and probably why it is much less efficient than fIX activation. The observation that fX and fV activation by fXIa are not Ca^{2+} -dependent (indeed, fV activation is faster in the absence of Ca^{2+}), suggest that fXa and fVa are produced during contact activation. This has implications for preparing plasma for assays that are sensitive to the activated forms of these factors. Failure to collect blood in a manner that limits contact activation (such as phlebotomy directly into a CTI-containing solution) may result in sufficient activation of fX and fV (and fVIII [10]) to affect results.

We did not find evidence that fXIa activated substrates further down the coagulation cascade from fV/fX. While fXIa readily cleaves prothrombin generating a species that runs in a similar position to α -thrombin on SDS-PAGE, the species lacks activity in a chromogenic assay, and fails to convert fibrinogen to fibrin (Supplemental Fig.1). FXIa does not convert fibrinogen to fibrin (not shown). Furthermore, fXIa does not convert fibrinogen to fibrin (not shown) or cleave factors II, IX or X (Supplemental Fig.2A), making it unlikely that this contact protease activated proteins downstream of fXI. FXIIa does cleave fV, however, it does not appear that fVa heavy or light chain are formed.

The observation that fXIa is a more promiscuous protease than originally suspected, may be relevant to its role in hemostasis and thrombosis. Despite the difference in bleeding phenotype, mice lacking fXI or fIX are comparably resistant to arterial thrombosis induced by FeCl_3 , consistent with the premise that fXIa activates fIX in this model [16,17]. However, mice lacking both fIX and fXI are more resistant than mice lacking only one of the factors. While this observation has only been made in one thrombosis model, the results do support the hypothesis that fXIa can act on targets other than fIX in some situations. Despite its modest role in hemostasis, there is mounting evidence for the thrombogenic potential of fXIa in humans. Plasma fXI levels correlate with risk of myocardial infarction [26], stroke [27], and venous thrombosis [28]. Initial attempts to use fXI concentrate to treat fXI deficiency were associated with a significant incidence of thrombosis [29,30], likely due to trace contamination with fXIa. FXI is a common contaminant in gamma globulin concentrates because IgG and fXI are difficult to separate chromatographically [31,32].

FXIa concentrations as low as 100 pM in gamma globulin are associated with thrombotic events [32]. Our data raise the possibility that the capacity of fXIa to activate plasma proteins in addition to fIX may contribute to its thrombogenic potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Anti-fXI IgGs

(A) Western blot of a mixture of reduced fXI and fXIa using Anti-XI^{AS} for detection. Positions of fXI (XI), fXIa heavy chain (XIa-HC), and fXIa catalytic domain (fXIa-CD) are indicated. (B) Cleavage of S-2366 by fXIa in the absence (○) or presence (●) of Anti-XI^{AS}. (C) Effects of Anti-XI^{A3} or Anti-IX^{AS} on the PTT of fXI-deficient plasma.

Figure 2. Clotting assays

PTTs of fIX-deficient (A) or fIX-depleted (B) plasma with control (C), Anti-IX^{Gla}, Anti-XI^{A3} or Anti-IX^{AS}. (C) Clotting time of fIX-deficient plasma supplemented with 3 nM fXIa and antibodies. (D) Clotting time of plasma from mice lacking fIX, fXI and fXII supplemented with human fXIa. Each circle indicates one clotting time. Bars indicate mean clotting time.

Figure 3. FV cleavage by fXIa

(A) Non-reducing SDS-PAGE of fV (150 nM) incubated with plasma fXIa (15 nM) in the presence or absence of 1.2 mM Ca²⁺. (B) SDS-PAGE of fV (500 nM) incubated with vehicle (left panel), or 50 nM fXIa^{WT}, fXIa/PKA3, or fXIa^{CD}. Right panel, fV incubated with fXIa^{WT} for 60 min with (+) or without (-) Anti-XI^{A3}. For panels A and B, minutes of incubation indicated on top, positions of mass standards (kDa) on the left, and positions of fV, fVa heavy chain (HC), fVa light chain (LC) and B-domain (BD) standards on the right. (C-E) Disappearance of fV (C), and appearance of fVa light chain (D) and B-domain (E) determined by densitometry of gels in Panel B. Symbols: (●) vehicle, (○) fXIa^{WT}, fXIa/PKA3 (◐) or fXIa^{CD} (◑). (F) Clotting times for normal (PNP) or fIX-deficient (fIX-DP) plasma supplemented with 30 nM fVa, 1.25 nM fXa or both. Each symbol indicates one clotting time. Bars indicate mean clotting time. (G) FV (30 nM) incubated with fXIa (15 nM). At various times, Va activity was determined as described in **Methods**.

Figure 4. FX cleavage by fXIa

(A) Non-reducing SDS-PAGE of fX (500nM) incubated with fXIa (125 nM). Positions of fX (X) and fXa (Xa) indicated at right. Mass standards in kDa are on the left. (B) FX (150 nM) incubated 1 hr with (+XIa) or without (-XIa) 15 nM fXIa, in the presence of vehicle (V), Anti-XI^{A3} (XI^{A3}) or aprotinin (Ap). Aliquots were added to fIX-deficient plasma (18.75 nM final concentration) and clotting was initiated with CaCl₂. Each circle indicates one clotting time. Bars indicate mean clotting time. (C) FX (150 nM) incubated with 15 nM plasma XIa (○), fXIa^{WT} (●), fXIa/PKA3 (□) or fXIa^{CD} (▪). FXa was measured by chromogenic assay. (D) Cleavage of S2765 (250 μM) by fX (150 nM) incubated 1 hr with 15 nM fXIa in the absence (C) or presence of divalent cations (1.2 mM Ca²⁺, 1 mM Mg²⁺ or 10 μM Zn²⁺). (E) FX (150 nM) incubated with fXIa (15 nM) and vehicle (○), Anti-IX^{Gla} (Δ) or anti-XI^{A3} (□). FXa was measured by prothrombinase assay. (F) Normal (○, □) or fIX-deficient (●, ▪) plasma incubated with PTT-A reagent. Aliquots were mixed with CTI, aprotinin and hirudin, with (□, ▪) or without (○, ●) apixaban. FXa was measured by chromogenic assay.

Figure 5. Thrombin generation

Thrombin generation (TG) in (A) fIX-deficient plasma supplemented with fXIa (0–15 nM) and 900 nM Anti-IX^{Gla}; (B) fIX-deficient plasma containing apixaban, or in fV deficient plasma initiated by 15 nM fXIa; (C) fIX-deficient plasma initiated by fIXaβ (10–900 pM). (D) Same as Panel C, with included 900 nM IgG Anti-IX^{Gla}; (E) fIX-deficient plasma supplemented with 900 nM IgG Anti-IX^{Gla} and 15 nM fXIa^{WT}, fXIa/PKA3 or control (C); (F) fIX-deficient plasma supplemented with 900 nM IgG Anti-IX^{Gla} and 15 nM fXIa^{WT} in the presence of control (C) or Anti-XI^{A3}. All plasmas were supplemented with 4μM CTI to inhibit fXIIa.

Figure 6. FeCl₃-induced carotid artery thrombosis

Occlusion of the carotid artery in C57Bl/6 mice was induced by applying FeCl₃ (2.5–15%) to the vessel. Groups of 10 WT (black bars), fXI^{-/-} (white), fIX^{-/-} (light gray) and fIX^{-/-}/fXI^{-/-} (dark gray) mice were tested at each FeCl₃ concentrations. Bars represent percent of mice with patent arteries 30 min after FeCl₃ exposure. For each FeCl₃ concentration, results marked with asterisks (*) are significantly different from WT for that concentration ($p < 0.05$).