THROMBOSIS AND HEMOSTASIS

FVIIa as used pharmacologically is not TF dependent in hemophilia B mice

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Activated factor VII is approved for treating hemophilia patients with autoantibodies to their factor IX or VIII; however, its mechanism of action remains controversial. Some studies suggest that FVIIa requires tissue factor (TF) for function and that the reason for the high dose requirement is that it must compete with endogenous FVII for tissue factor. Others suggest that FVIIa binds platelets where it activates FX directly; the high concentration required would result from FVIIa’s weak affinity for phospholipids. We address this question by infusing a chimera of mouse FIX (Gla and EGF1) with FVIIa (EGF2 and catalytic domain) into hemophilia B mice. This mutant has no TF-dependent activity because it cannot functionally bind TF at physiologically relevant concentrations. In vivo, this mutant is as effective as mouse FVIIa in controlling bleeding in hemophilia B mice. Our results suggest that the hemostatic effect of pharmacologic doses of FVIIa is TF independent. (Blood. 2014;123(11):1764-1766)

Introduction

Human coagulation factor VIIa is approved to treat bleeding in hemophilia patients with inhibitors. Two theories attempt to explain why high doses of FVIIa are required for hemostasis. The tissue factor (TF)-dependent mechanism suggests that FVIIa’s hemostatic effect requires its binding to TF, which is expressed on cell surfaces at the site of injury.1,2 In this scenario, the high concentration of FVIIa must compete with endogenous FVII for TF. The alternative phospholipid-dependent mechanism postulates that FVIIa binds phospholipids on the platelet surface and activates factor X. Because FVIIa has low affinity for phospholipid, a high concentration of FVIIa is required to achieve hemostasis.3,4 Related to the platelet-binding mechanism, the possibility that platelet GP1bα binds FVIIa and contributes to its TF-independent activity has also been proposed.5

Recently, Shibeko et al6 used in vitro data and mathematical modeling and concluded that both mechanisms contribute to enhanced thrombin generation but that the TF-dependent mechanism dominates.

To test whether the TF pathway is dominant, we used a chimeric FVIIa molecule, mFIXgla-egf1FVIIa, with minimal affinity for TF. FVIIa is a multidomain enzyme containing an amino-terminal γ-carboxyglutamic acid–rich (Gla) domain, 2 epidermal growth factor (EGF)-like domains, and a trypsin-like serine protease domain. Previous studies indicate that the Gla domain, the first EGF domain (EGF1), and the protease domain all contribute to TF binding.7,8 Furthermore, our unpublished observations indicate that the affinity of human FIXgla-egf1FVIIa for TF is reduced about 600 000-fold compared with wtFVIIa. Our chimeric FVIIa molecule, mFIXgla-egf1FVIIa, has had its Gla and EGF1 domains replaced by those of mouse FIX (which has a similar domain structure); its functional affinity for TF is reduced to unmeasurable levels, yet its ability to catalyze the conversion of FX to FXa in the presence of Ca2+ and phospholipid is similar to that of FVIIa. If FVIIa, as used pharmacologically, is active only when in a functional complex with TF, then mFIXgla-egf1FVIIa, with its much-reduced TF-binding activity, should not provide hemostasis in the hemophilia B mouse; if, however, mFVIIa works directly on platelet surfaces, then the molecules with reduced TF binding (such as mFIXgla-egf1FVIIa) should stop bleeding as well as FVIIa.

Study design

Mouse complementary DNAs were cloned and purified as previously described.9,10 Insertion of the short amino acid sequence RKRRKR between R193 and I194 was performed as described previously so that secreted FVIIa is activated when secreted.11

TF-dependent FX activation used human TF (Innovin; DADE/ Behring, Deerfield, IL) or mouse TF–containing microparticles.12 Binding was inferred by the ability of the complex to convert FX to FXa, as measured by its ability to cleave Spectrozyme FXa (American Diagnostica).8

Clotting activity used a standard curve with NovoSeven (Novo Nordisk, Bagsvaerd, Denmark). Prothrombin time (PT) assays used human FVII–deficient plasma; activated partial thromboplastin time (APTT) assays used human FIX–deficient plasma (both from George King Bio-Medical, Overland Park, KS) using a START 4 Coagulation Analyzer (Diagnostics Stago, Asnieres, France).

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Animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina. All experiments used 6- to 10-week-old hemophilia B mice on a C57BL/6 background. Saphenous vein bleeding was performed as described elsewhere. The saphenous vein model is sensitive to TF. The average time to hemostasis (ATTH) in homozygous mice with low TF (Parry et al) was doubled, relative to either wild-type (WT) mice or heterozygous littermates (data not shown). This result is consistent with tail-bleeding studies in low-TF mice.

Results and discussion

We did 2 tests to ensure that FIXgla-egf1FVIIa is not TF dependent. First, we examined the intrinsic and extrinsic coagulation activity of mFVIIa and mFIXgla-egf1FVIIa in TF-independent APTT assays (Figure 1A) and TF-dependent PT assays (Figure 1B). Mouse FIXgla-egf1FVIIa has the same APTT activity as does mFVIIa ($P = .63$) (Figure 1A); however, mFIXgla-egf1FVIIa has no PT activity when compared with mFVIIa (Figure 1B), which indicates that mFIXgla-egf1FVIIa does not bind to TF.

Second, we measured whether mFIXgla-egf1FVIIa catalyzes the conversion of FX to FXa. Mouse FVIIa binds to human TF in a concentration-dependent manner to catalyze the conversion of FX to FXa. However, mFIXgla-egf1FVIIa exhibits no measurable TF-dependent FXa generation (Figure 1C). Similarly, mFVIIa is functional with mouse TF-bearing microparticles, but mFIXgla-egf1FVIIa is not (Figure 1D).

The effect of mFVIIa on hemostasis in the hemophilia B mouse was examined by the saphenous vein bleeding model (Figure 2B). Three different concentrations of mFVIIa or mFIXgla-egf1FVIIa were infused into hemophilia B mice 10 minutes before saphenous vein incision; the time to hemostasis for each bleeding event was recorded over a 30-minute time period and the ATTH for each mouse was calculated. Control WT C57BL/6 mice (n = 4) had a median ATTH of 44 seconds; hemophilia B mice (n = 3) failed to achieve hemostasis within the 30-minute experimental time frame. The median ATTH remained similar for mFVIIa- and mFIXgla-egf1FVIIa-treated hemophilia B mice at the 3 tested doses.

Clearly, FIXgla-egf1FVIIa does not productively bind TF; however, there are other differences in these molecules that may affect their function. For example, FIX’s Gla domain alone is sufficient for binding to collagen IV in the subendothelium. Thus, it is possible that...
modified pharmacokinetics of FVIIa compared with FIXgla-egf1FVIIa due to additional interactions within the mouse circulation may explain some of our data.

Hemophilia B mice require much higher doses of FVIIa than do humans to correct bleeding. Our 2-mg/kg dose is higher than the 90- to 270-µg/kg dose currently recommended for human use; however, it is consistent with others’ observations.18 For example, Ivanciu et al report that 5 mg/kg of mouse or human FVIIa prevents bleeding equally well in a hemophilia B mouse.18 Because the affinity of human FVIIa for mouse TF is 1000-fold less than that of mFVIIa for mouse TF, this is further evidence that pharmacologic use of FVIIa does not require TF for hemostasis.19

In summary, FIXgla-egf1 does not productively bind TF but is as effective as mFVIIa in promoting hemostasis in a hemophilia B mouse. Blood-cell–derived TF is the decisive trigger for the massive fibrin deposition of deep vein thrombosis.20 Moreover, long-term high levels of FVIIa expression in mice cause premature death related to thrombosis in the lung and heart, both tissues rich in TF.11 Thus, because FIXgla-egf1FVIIa does not bind TF, it is likely a safer and more effective molecule than FVIIa, especially for those cases where FVIIa is used off-label for uncontrolled bleeding.

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Authorship

Contribution: All authors contributed to the writing and correction of the manuscript, and most of the experimental work was done by D.F.

Conflict-of-interest disclosure: The authors declare no competing financial interests. A patent has been filed for the use of the chimera for treating patients with inhibitors.

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