Influence of emicizumab on protein C-mediated clotting regulation

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ABSTRACT

Emicizumab, a FVIII-mimetic bispecific antibody, is insensitive to degradation by activated protein C (APC) and may thus induce a procoagulant state. We investigated the effect of emicizumab on protein C-mediated inhibition of coagulation under in vitro conditions mimicking physiological and pathological clotting activation. Thrombin generation (TG) in tissue factor-triggered hemophilic plasma containing emicizumab (50 µg/mL) was inhibited by APC or thrombomodulin in a concentration-dependent manner, and to a similar extent as in plasma added with FVIII (Kovaltry, 1 IU/mL). However, when clotting was activated via the intrinsic pathway, emicizumab-plasma displayed resistance to APC, manifested by a barely detectable prolongation of the lag time of TG, and by the lack of inhibition of FXa generation. Moreover, in contact-activated plasma added with APC, the generation of a second wave of thrombin, following prothrombin replenishment, was much greater in emicizumab-plasma than in Kovaltry-plasma, suggesting that the insensitivity of emicizumab to APC may translate in greater thrombin formation. Considering the major role played by the contact system in the thrombotic process, hemophilia A patients under emicizumab treatment might be at increased thrombotic risk.

Introduction

Emicizumab (Hemlibra) is a FVIII-mimetic bispecific antibody licensed for the prevention of bleeding in hemophilia A patients with and without inhibitors. It mimics the function of FVIII by simultaneously binding FIXa and factor X, thereby promoting the co-localization of the enzyme-substrate complex on the phospholipid surface. Compared to FVIII, emicizumab presents several differences from biochemical and mechanistic perspectives. FVIII is highly regulated as it needs to be activated by thrombin to express its full cofactor activity and, once activated (FVIIIa), it is degraded by activated protein C (APC), which represents one of the major physiological control mechanisms of coagulation. Moreover, FVIIIa activity decays spontaneously as a result of A2 subunit dissociation. In contrast, emicizumab is constitutively active, is stable, and cannot be inactivated by APC, and therefore it might tilt the coagulation balance towards a procoagulant state.

APC is a serine protease, derived from the activation of protein C (PC) by the thrombin-thrombomodulin complex, which downregulates thrombin generation by inactivating the cofactors FVa and FVIIIa through limited proteolysis at specific arginine residues. The best-known condition associated with refractoriness to APC is the FV Leiden mutation, which, in rare homozygous carriers, increases the thrombotic risk by approximately 80-fold. In the physiological clotting cascade, FVIIIa serves as cofactor of FIXa in the intrinsic tenase complex whereas FVa is part of the prothrombinase complex, which leads to the generation of the final clotting enzyme thrombin. Moreover, FV is able to function as cofactor of APC in the inactivation of FVIIIa. In view of the different roles of the two cofactors, a key question is whether APC-resistant FVIII has the same effect on thrombin generation and thrombotic risk as APC-resistant FV. Wilhelm et al. reported that a FVIII variant (FVIII-R336Q/R562Q) insensitive to degradation by APC made FVIII-
deficient plasma resistant to the anticoagulant effect of APC and displayed a greater prothrombotic activity than wild-type B-domainless FVIII in a ferric chloride thrombosis model in hemophilia A mice. Moreover, acquired APC resistance is a risk factor for venous thrombosis, and one of the mechanisms behind this form of APC resistance is the elevation of FVIII levels. A finding that provides a likely explanation for the increased thrombosis risk associated with high FVIII levels. Based on these observations, it is conceivable to hypothesize that the replacement of FVIII with emicizumab will impair the anticoagulant activity of PC, thereby increasing thrombin generation and, eventually, the thrombotic risk.

To date, only one study has specifically investigated the effect of emicizumab on APC-dependent control of coagulation. The authors reported that APC addition to hemophilic A plasma supplemented with emicizumab (referred to as emicizumab-plasma) resulted in a concentration-dependent reduction of thrombin generation, which was entirely due to the inactivation of FV(a). The study, however, does not allow us to safely conclude that APC-dependent inhibition of coagulation is not impaired by emicizumab for the following reasons. First, emicizumab-plasma was not compared to a proper control consisting of the same FVIII-deficient plasma supplemented with a “traditional” FVIII preparation (plasma-derived or recombinant). Therefore, it is not possible to establish to what extent the inhibition of coagulation by APC in emicizumab-plasma compares to that observed in a plasma containing a FVIII sensitive to APC degradation. Second, as recognized by the same authors, the concentration of emicizumab added to FVIII-deficient plasma (30 μg/mL) was lower than that achieved in patients (40-60 μg/mL) and thus it is unknown if greater concentrations of emicizumab would reduce the anticoagulant response to APC. Finally, the thrombin generation experiments were performed using a single condition in which APC was added to plasma triggered with a combination of tissue factor (TF) and ellagic acid.

Our study was undertaken to further evaluate the influence of emicizumab on the inhibition of thrombin generation by the PC system, using experimental conditions that mimic both physiological and pathological clotting activation. Our data suggest that, under specific conditions, the resistance of emicizumab to APC results in faster and greater thrombin generation that may induce a hypercoagulable state.

Materials and Methods

Hemophilia A plasma and reagents

Frozen Hemophilia A plasma (FVIII<1%, 1 mL per vial) was purchased from George King Bio-Medical (Overland Park, KS, USA). Three different batches of hemophilic plasma were used in our study.

Emicizumab was obtained from Roche (Basel, Switzerland), Kovaltry (recombinant full-length FVIII) from Bayer (Milan, Italy), and Beriate (plasma-derived FVIII) from CSL Behring (Marburg, Germany).

The following reagents were purchased from the indicated sources: aPTT reagent (Synthasil), human thromboplastin (Recombiplastin), and S-2732 (FXa-specific substrate) from Instrumentation Laboratory (Milan, Italy); reptilase from Bothrops atrox venom, fluorogenic thrombin substrate Z-Gly-Gly-Arg-7-amino-4-methylcoumarin (ZGGR-AMC) and thrombin calibrator from Diagnostica Stago (Asnieres, France); human APC (Xigris) from Eli Lilly (Indianapolis, IN, USA); soluble rabbit thrombomodulin (TM) from Sekisui Diagnostics (Darmstadt, Germany); hirudin from Abbott GmbH (Ludwigshafen, Germany); purified human FXIIIa from Life Technologies (Monza, Italy); purified human prothrombin from Haematologic Technologies (Essex Junction, VT, USA); bovine fibrinogen from Sigma (Milan, Italy). Phospholipid vesicles (20% phosphatidylserine, 60% phosphatidylcholine, 20% phosphatidylethanolamine; Avanti Polar Lipids, Alabaster, AL, USA) were prepared by sonication.

Fluorogenic thrombin generation assay

All reported concentrations are final concentrations per mL of plasma (emicizumab, FVIII concentrate, APC, TM) or per mL of the final mixture. Thrombin generation (TG) was evaluated according to Hemker et al., with minor modifications as follows. Forty μL plasma, 5 μL emicizumab (50 μg/mL) or Kovaltry (1 IU/mL), 2.5 μL phospholipids (5 μg/mL), 5 μL buffer, APC or TM (at the indicated concentrations), and 2.5 μL clotting trigger were added to round-bottom 96-well plates (Immulon 2HB; Dynex Technologies). The reaction was started by the addition of a 10 μL mixture containing CaCl2 and ZGGR-AMC (16 mM and 0.42 mM, respectively). Measurements were taken every 20 seconds in a FluoroScan Ascent fluorometer (Thermo Scientific, Dreieich, Germany), and data were analyzed using the Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands). The TG parameters evaluated in this study were lag time, thrombin peak, and endogenous thrombin potential (ETP). All samples and calibrators were run in duplicate.

Unless otherwise specified, coagulation was triggered by thromboplastin (1:10000 final dilution, corresponding to approximately 1 pM tissue factor) or human FXIa (10 pM). In some experiments, the intrinsic pathway of coagulation was activated with 1 μL diluted aPTT reagent or Kovaltry replaced by Beriate (1 IU/mL). The effect of APC or TM on TG parameters was calculated as the ratio between the values recorded in the presence and in the absence of APC or TM. Thus, as for lag time, the greater the ratio the stronger the anticoagulant effect of APC, whereas for Peak and ETP, the lower the ratio the stronger the APC effect.

Functional thrombin generation assay

To evaluate the effect of prothrombin replenishment (after prothrombin consumption) in emicizumab- and Kovaltry-plasma, thrombin generation in the absence and in the presence of APC was assessed by a two-stage clotting assay as follows. Six-hundred-forty μL prewarmed hemophilic plasma, freshly defibrinated as reported, were added into a test tube placed in a water bath at 37°C, followed by 20 μL emicizumab or Kovaltry (50 μg/mL and 1IU/mL, respectively), 20 μL phospholipids (5 μg/mL), 40 μL APC (0.4 μg/mL) or buffer, and 80 μL FXIa (10 nM). The reaction was started by the addition of 160 μL CaCl2 (16 mM). Then, 60 μL aliquots were transferred at intervals in a prewarmed tube prefilled with 30 μL PBS containing bovine fibrinogen (2 mg/mL) and sodium citrate (0.036 M), and the clotting time was assessed manually by the tilting method. Thrombin activity in each subsample was calculated by refer-
ence to a calibration curve constructed with increasing concentrations of purified human thrombin. After thrombin activity had fallen to near zero, prothrombin (100 µg/mL) was added to the reaction mixture, and the new thrombin formed was monitored using the same two-stage assay.

FXa generation assay

FXa generation was measured by a chromogenic assay as follows. Fifteen µL defibrinated hemophilic plasma, 5 µL emicizumab (50 µg/mL) or Kovaltry (1 IU/mL), 5 µL phospholipids (5 µg/mL), 5 µL buffer or APC (0.4 µg/mL), 15 µL FXIa (10 pM), 15 µL hirudin (200 U/mL) or buffer and 50 µL S-2732 (FXa-specific substrate, 1.6 mM) were added to round-bottom 96-well plates, after which the reaction was started by the addition of 10 µL CaCl₂ (6 mM). Substrate cleavage was monitored by the change in absorption at 405 nm in a microplate reader (Multiskan FC; Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

Results are expressed as mean ± SD. The difference between emicizumab- and Kovaltry-plasma samples, which were always run in parallel, was assessed by paired Student t-test using the MedCalc software (Mariakerke, Belgium). Two-tailed P<0.05 was considered statistically significant.

Results

Effect of emicizumab on thrombin generation in tissue factor-triggered hemophilic plasma

Experiments were carried out using a low concentration of TF (1 pM) so that the intrinsic tenase complex, generated mainly through the Josso loop (FIX activation by TF/FVIIa), contributed significantly to thrombin formation, as testified by the very little thrombin formation in the absence of FVIII addition (not shown). Typical TG curves in the presence of increasing concentrations of APC are illustrated in Figure 1A. APC-induced changes in TG parameters, calculated as APC ratio, showed that there was a concentration-dependent inhibition of TG in both emicizumab- and Kovaltry-plasma, as indicated by the progressive prolongation of lag time and reduction in ETP and thrombin peak (Figure 1B). Although the changes in emicizumab-plasma were slightly less pronounced than in Kovaltry-plasma, the dif-

Figure 1. Effect of increasing concentrations of activated protein C (APC) on tissue factor-triggered thrombin generation in hemophilic plasma supplemented with emicizumab (50 µg/mL) or Kovaltry (1 IU/mL). A) Typical thrombin generation curves in emicizumab-plasma (left) and Kovaltry-plasma (right); B) APC-induced changes in thrombin generation parameters calculated as APC ratio (value in the presence of APC divided by value without APC). For lag time, the greater the ratio the stronger the anticoagulant effect of APC; for Peak and endogenous thrombin potential (ETP), the lower the ratio, the stronger the APC effect. Data are the mean ± standard deviation of 6 independent experiments.
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Comparisons between the two samples were not significant for any TG parameters. Qualitatively similar results were obtained when thrombin generation was performed in the presence of different concentrations of TM (Supplementary Figure 1) or when Kovaltry was replaced by Beriate (1 IU/mL), a plasma-derived FVIII concentrate (not shown).

Effect of emicizumab on thrombin generation in contact-activated hemophilic plasma

To make FXa generation exclusively dependent on FVIII (intrinsic tenase), we activated plasma with FXIa (10 pM) or diluted aPTT reagent (1/50 final dilution). Figure 2 illustrates the results with FXIa-triggered plasma. In the absence of APC or TM addition, emicizumab-plasma displayed a significantly shorter lag time compared to Kovaltry-plasma (2.8±0.81 vs. 5.1±0.84 min, P<0.001) most likely because emicizumab, contrary to FVIII, does not need to be activated to function as co-factor of FIXa. Upon addition of APC, the lag time prolongation was markedly and significantly less pronounced in emicizumab-plasma than in Kovaltry-plasma at all tested APC concentrations. As to the reduction of ETP and thrombin peak, a significant difference between emicizumab and Kovaltry samples was only seen at the highest APC concentration (Figure 2). When the TG assay was performed in the presence of two concentrations of TM (15 and 30 nM), we confirmed a striking difference in TM-induced lag time prolongation between emicizumab and Kovaltry but did not find significant differences in Peak and ETP reduction at any TM concentration (Supplementary Figure 2).

Qualitatively similar results were obtained upon the addition of APC or TM to plasma samples activated with diluted aPTT reagent (not shown).

Based on the above findings, all following experiments were performed in contact-activated hemophilic plasma added with APC.

Effect of emicizumab on FXa generation

FXa generation was evaluated in defibrinated plasma using a chromogenic substrate (S-2732). The time course of substrate cleavage (optical density [OD] increase) in plasma challenged with FXIa showed remarkable differences between emicizumab and Kovaltry-plasma (Figure 3). In the absence of APC, FXa formation in emicizumab-plasma started earlier and increased more rapidly than in Kovaltry-plasma; moreover, as expected, FXa generation in the presence of APC was marginally changed in emicizumab-plasma but markedly reduced in Kovaltry-plasma (Figures 3A and B). To evaluate the possible contribution of thrombin to FXa generation (via FVIII activation) and sub-

![Figure 2. Effect of increasing concentrations of activated protein C (APC) on FXIa-triggered thrombin generation in hemophilic plasma supplemented with emicizumab (50 µg/mL) or Kovaltry (1 IU/mL). A) Typical thrombin generation curves in emicizumab-plasma (left) and Kovaltry plasma (right); B) APC-induced changes in thrombin generation parameters calculated as APC ratio (see legend to Figure 1 for further details). Data are the mean ± standard deviation of 6 independent experiments. *p<0.05 by paired Student t-test.](image-url)
strate cleavage, the assay was also performed in the presence of hirudin (200 U/ml). Under this condition, the OD increase in emicizumab-plasma was only slightly reduced compared to the sample without hirudin, and the little difference between the absence and presence of APC became even smaller (Figure 3C), suggesting a minimal contribution of thrombin to substrate cleavage. On the contrary, in Kovaltry-plasma, substrate cleavage was markedly reduced in the absence of APC and practically absent in its presence (Figure 3D), a finding consistent with the key role played by thrombin in FVIII activation and, consequently, in FX activation by the intrinsic tenase complex.

**Effect of emicizumab on the second burst of thrombin generation**

The experiments with contact-activated plasma indicate that, in the presence of APC, the accumulation of FXa in emicizumab-plasma was much greater than in Kovaltry-plasma, which is expected given the insensitivity of emicizumab to degradation by APC. Because in our *in vitro* assay FX activation by FXIIa persisted after the disappearance of thrombin, we wished to determine how the difference in FXa accumulation between emicizumab- and Kovaltry-plasma would impact thrombin generation after reconstitution of prothrombin levels, thereby mimicking the *in vivo* conditions in which prothrombin exhaustion is very unlikely to occur. For that purpose, we used FXIIa-triggered (defibrinated) plasma containing APC (0.4 µg/mL) and determined the generation of thrombin by a clotting assay on subsamples taken at intervals. Once thrombin activity fell to near zero, we added purified prothrombin (100 µg/mL) and evaluated the second wave of thrombin generation by the same clotting assay. As shown in Figure 4, the first thrombin curve of emicizumab-plasma showed a visibly shorter lag time than Kovaltry-plasma but a similar thrombin peak. On the contrary, the second curve displayed more marked differences, the rate and the amount of thrombin formed in emicizumab-plasma being much greater than those recorded in Kovaltry-plasma. No new thrombin formation was detected if the FXa inhibitor rivaroxaban (10 µg/mL) was added along with purified prothrombin (not shown).

**Discussion**

Our study shows that hemophilic plasma supplemented with emicizumab displays, under certain conditions, a weaker response to the anticoagulant effect of APC or TM addition compared to APC-sensitive FVIII preparations (Kovaltry and Beriate). The response to the PC system was investigated by a thrombin generation assay, which was performed using as coagulation trigger either TF or an activator of the contact system (FXIIa or diluted aPTT reagent). The concentrations of emicizumab and FVIII concentrates added to hemophilic plasma (50 µg/mL and 1 IU/mL, respectively) had a comparable effect on thrombin generation parameters, except for a shorter lag time of contact-activated plasma supplemented with emicizumab (Figure 2). As to the response to the PC system, calculated as APC ratio or TM ratio, we had different results depending on

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**Figure 3.** Effect of activated protein C (0.4 µg/mL) on FXa generation in FXIIa-triggered hemophilic plasma supplemented with emicizumab (50 µg/mL, panels A and C) or Kovaltry (1 IU/mL, panels B and D). FXa generation was assessed in defibrinated plasma by a chromogenic assay. The curves represent optical density at 405 nm (OD405 nm) increase over time. In panels C and D, experiments were performed in the presence of the thrombin inhibitor hirudin (200 U/mL). Curves from an experiment representative of 4 independent experiments with similar results are depicted.

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The coagulation trigger. In TF-triggered coagulation, the changes in thrombin generation parameters recorded in emicizumab-plasma were fairly comparable to those observed in Kovaltry-plasma (Figure 1). On the contrary, when coagulation was activated via the contact system, we found that the lag time prolongation induced by APC or TM addition to emicizumab-plasma was markedly and significantly smaller than that observed in Kovaltry-plasma at all tested concentrations of APC and TM. This notwithstanding, no significant differences were observed between emicizumab and Kovaltry samples as to the reduction of ETP and thrombin peak by APC or TM, except for the highest APC concentration. This finding might suggest that the inactivation of FVIII by APC plays a major role in controlling the amount of thrombin formation.

To further explore the influence of emicizumab on the control of coagulation by the PC system, we determined the generation of FXα in contact-activated plasma and found that, upon addition of APC, FXα formation was virtually unaffected in emicizumab-plasma but markedly reduced in Kovaltry-plasma. These results are well expected because, in contact-activated plasma, FXα can only be generated through the intrinsic tenase, whose activity is strictly dependent on the availability of FVIIIa. Therefore, given that the FVIII-mimetic emicizumab is constitutively active and insensitive to degradation by APC, FXα generation will be the same regardless of the presence of APC.

An intriguing observation about the experiments with contact-activated plasma containing APC is that, despite the much greater generation of FXα in emicizumab-plasma, the only TG parameter that consistently showed a weaker response to APC, compared to Kovaltry-plasma, was the lag time. One feature of our TG assay is that prothrombin is consumed rather quickly, whereas FXα formation continues beyond thrombin disappearance (compare Figure 2 with Figure 3), which is at variance with the in vivo situation where prothrombin exhaustion is very unlikely to occur. It is possible, therefore, that our in vitro model (closed system) did not allow us to fully appreciate the real impact of FXα formation on thrombin generation. In an attempt to overcome this limitation, we performed the TG assay by adding purified human prothrombin soon after endogenous thrombin had fallen to near zero, giving rise to two TG curves. In the presence of APC, while the first TG curve recapitulated the results obtained with the fluorogenic assay, the second one showed a marked difference between emicizumab- and Kovaltry-plasma, in that the amount of thrombin generated in the former was significantly greater than that recorded in the latter, suggesting that the different accumulation of FXα has the potential to influence not only the timing but also the amount of thrombin formation. Thus, at variance with Yada et al.,14 we were able to show that, under specific experimental conditions, emicizumab attenuates the anticoagulant effect of PC thereby increasing thrombin generation and purportedly the thrombotic risk.

Conclusions

Safety concerns for an increased risk of thrombotic complications in patients with hemophilia A under treatment with emicizumab have been raised by the pivotal HA VEN 1 trial,1 which reported six episodes of thrombosis in patients with inhibitors concomitantly treated with bypassing agents. Therefore, even though no evidence has been provided since then of emicizumab-related thrombosis, the resistance of emicizumab to APC is perceived as potentially prothrombotic and has led investigators to develop a next-generation FVIII-mimetic antibody equipped with a self-regulation mechanism that inactivates the antibody when enough thrombin is generated.17 Our in vitro study provides experimental evidence suggesting that emicizumab may indeed tilt the coagulation balance towards a procoagulant state by attenuating the anticoagulant effect of the PC system when coagulation is activated via the intrinsic pathway. This finding is clinically relevant because the activation of the contact system by cell-derived molecules, such as DNA and polyphosphate, has been shown to play a major role both in venous and arterial thrombosis.18,19 Further studies in samples from hemophilic patients under treatment with emicizumab are warranted to confirm our results with spiked plasma samples.

References

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Online supplementary material:
Figure S1. Effect of increasing concentrations of thrombomodulin (TM) on tissue factor-triggered thrombin generation in hemophilic plasma supplemented with emicizumab (50 µg/mL) or Kovaltry (1 IU/mL).
Figure S2. Effect of two different concentrations of thrombomodulin (TM) on FXIa-triggered thrombin generation in hemophilic plasma supplemented with emicizumab (50 µg/mL) or Kovaltry (1 IU/mL).