Coagulation factor VII variants resistant to inhibitory antibodies

Alessio Branchini\(^1\); Marcello Baroni\(^1\); Caroline Pfeiffer\(^2,3\); Angelika Batorova\(^4\); Muriel Giansily-Blaizot\(^6\); Jean F. Schved\(^2\); Guglielmo Mariani\(^1\); Francesco Bernardi\(^1\); Mirko Pinotti\(^4\)

\(^1\)Department of Life Sciences and Biotechnology and ITTA Centre, University of Ferrara, Italy; \(^2\)Department of Biological Haematology, CHU of Montpellier, Montpellier, France; \(^3\)UMR 3145 SysDiag CNRS/IRIS-Rad, Parc Euromédecine, Montpellier, France; \(^4\)Department of Haematology and Transfusion Medicine, National Haemophilia Centre, University Hospital, Bratislava, Slovakia

**Summary**

Replacement therapy is currently used to prevent and treat bleeding episodes in coagulation factor deficiencies. However, structural differences between the endogenous and therapeutic proteins might increase the risk for immune complications. This study was aimed at identifying factor (F)VII variants resistant to inhibitory antibodies developed after treatment with recombinant activated factor VII (rFVIIa) in a FVII-deficient patient homozygous for the p.A354V-p.P464HFs mutation, which predicts trace levels of an elongated FVII variant in plasma. We performed fluorescent bead-based competition, ELISA-based competition as well as fluorogenic functional (activated FX and thrombin generation) assays in plasma and with recombinant proteins. We found that antibodies displayed higher affinity for the active than for the zymogen FVII (half-maximal binding at 0.54 ± 0.04 and 0.78 ± 0.07 BU/ml, respectively), and inhibited the coagulation initiation phase with a second-order kinetics. Isotypic analysis showed a polyclonal response with a large predominance of IgG1. We hypothesised that structural differences in the carboxyl-terminus between the inherited FVII and the therapeutic molecules contributed to the immune response. Intriguingly, a naturally-occurring, poorly secreted and 5-residue truncated FVII (FVII-462X) escaped inhibition. Among a series of truncated rFVII molecules, we identified a well-secreted and catalytically competent variant (rFVII-464X) with reduced binding to antibodies (half-maximal binding at 0.198 ± 0.003 BU/ml) as compared to the rFVII-wt (0.032 ± 0.002 BU/ml), which led to a 40-time reduced inhibition in activated FX generation assays. Taken together our results provide a paradigmatic example of mutation-related inhibitory antibodies, strongly support the FVII carboxyl-terminus as their main target and identify inhibitor-resistant FVII variants.

**Keywords**

Inhibitory antibodies, replacement therapy, factor VII deficiency, carboxyl-terminus, recombinant proteins

**Correspondence to:**
Prof. Francesco Bernardi
Department of Life Sciences and Biotechnology, University of Ferrara
Via Fossato di Mortara 74, 44121 Ferrara, Italy
Tel: +39 0532 974425, Fax: +39 0532 974484
E-mail: ber@unife.it

* A. Branchini and M. Baroni contributed equally to this study.

**Introduction**

The formation of inhibitory antibodies against the infused coagulation factor represents the most severe complication of replacement therapy in inherited haemorrhagic disorders. Whereas it occurs in approximately 30% of patients with severe haemophilia A (HA), this complication affects a minor proportion (3%) of haemophilia B (HB) patients (1–5). At variance from haemophilia, little is known about inhibitory antibodies developed in patients with rare bleeding disorders such as deficiency of factor VII (FVII) (6–9), which shares high similarity with factor IX (FIX) at the gene and protein level (10, 11). Taking into account the key role of FVII in triggering blood coagulation, its complete deficiency does not appear to be compatible with life, as indicated by the extreme rarity of patients with null genetic conditions (http://www.hgmd.cf.ac.uk; http://www.isth.org) (12) and by fatal perinatal bleeding in F7 knock-out mice (13). These FVII-specific features, both functional and genetic, explain why scanty information are available on anti-FVII inhibitory antibodies in patients.

Over years, enormous efforts have been made in haemophilies to elucidate the molecular and cellular mechanisms underlying the immune response to therapeutic proteins and to identify predictive risk factors. The inhibitor development depends on the interaction of multiple non-genetic and genetic risk factors (14–17), among those the causative gene lesions, with the so-called null mutations being associated to the highest incidence of inhibitors in the affected patients (2, 4, 16). Notwithstanding, inhibitors have been also found in HA patients with residual protein and activity levels in plasma, as clearly indicated by the inhibitor occurrence in mild forms (18–20). Differently, there is little evidence for HB
patients with residual FIX levels who developed inhibitors. Intriguingly, we characterised anti-FVII inhibitory antibodies developed after treatment with recombinant activated FVII (rFVIIa, Novo Nordisk) in a FVII-deficient patient with residual circulating FVII produced by the most frequent F7 mutation in Central Europe (12, 21, 22), the F7 p.A354V-p.P464Hfs. By a rationale mutation-driven design followed by functional and binding assays, we provide a paradigmatic example of i) mutation-related inhibitory antibodies recognising the protein carboxyl-terminus, which would stem from the structural differences between the endogenous variant and the infused therapeutic molecules, and of ii) efficiently secreted and inhibitor-resistant recombinant FVII molecules, characterised by a truncated carboxyl-terminus.

Materials and methods

This study was carried out within the frame of the Seven Treatment Evaluation Registry (STER) overseen by the STER Study Group that was approved by the Ethic Committee of L’Aquila University (coordinator’s [Prof. G. Mariani] institution) and, in parallel, by the Committees of the other institutions involved.

Plasma samples

For functional and binding assays we used the following plasma samples:

- i) plasma from the patient homozygous for the F7 p.A354V-p.P464Hfs mutation (9) with a titre of 32 Bethesda Units/ml (BU/ml), being 1 BU the amount of inhibitors able to neutralise 50% of coagulation factor activity (23),
- ii) plasma from a patient homozygous for the F7 p.R462X nonsense mutation, causing low levels (0.8% of normal) of circulating FVII molecules lacking the last 5 carboxyl-terminal residues (FVII-462X), and displaying gain-of-function features (24),
- iii) Pooled normal plasma (PNP, Hyphen BioMed, Andresy, France),
- iv) FVII-deficient plasma (George King, Bio-Medical Inc., Overland Park, KS, USA).

Recombinant FVII variants

The recombinant FVII (rFVII) variants were produced by transient transfection of baby hamster kidney (BHK) cells, as previously described (24).

ELISA-based binding and competition assays

Binding studies were adapted from a previously described protocol (25). 10 nmol/l (1 pmol/well) rFVIIa (NovoSeven, NovoNordisk®, Baegsvard, Denmark) or plasma-derived FVII (pdFVII, Haematologic Technologies Inc., Essex Junction, VT, USA) in 50 mmol/l carbonate (pH 9.2) were incubated for 16 hours (h) at 4°C on a 96-well microplate (Nunc MaxiSorp®, Roskilde, Denmark). After blocking with 6% bovine serum albumin (BSA) in phosphate-buffered saline (PBS, pH 7.4), 0.05% Tween-20, serial dilutions of patient plasma were incubated for 1 h at 37°C. Bound antibodies were detected by a goat HRP-conjugated anti-human IgG (Bethyl Laboratories, Montgomery, TX, USA).

Competition assays essentially exploited the same protocol. Preliminary studies with varying concentrations of rFVIIa or pdFVII, either coated or as competitors, and of patient plasma containing the antibodies, led us to optimise the experimental conditions. pdFVII (1 pmol/well) was incubated with anti-FVII antibodies (0.2 BU/ml) alone or in the presence of increasing concentrations (0.125–4 nmol/l) of rFVIIa, pdFVII, or of activated pdFVII (pdFVIIa) as competitors.

Studies on fluorescent beads

The assays were based on the x-MAP™ technology (26). Functionalisation of activated magnetic fluorescently-tagged carboxyl microbeads (1.25 × 10⁶/ml beads #46, Bio-Rad Laboratories, Hercules, CA, USA) was performed by incubation for 2 h at room temperature with 9 µg of an anti-FVII monoclonal antibody (mAb, American Diagnostica Inc., Greenwich, CT, USA) or 6 µg of rFVIIa diluted in 50 mmol/l 2-N-morpholinoethanesulfonic acid (MES), pH 6.0 (Sigma, St.Louis, MO, USA). The final count of rFVIIa- and mAb-coupled beads was 6.6 × 10⁶ and 5.5 × 10⁶ beads, respectively.

Binding of antibodies in patient plasma to recombinant FVII variants

mAb-coupled beads were diluted in PBS (pH 7.4) with 1% BSA to a final count of 3,000 beads/well. Beads were incubated with the rFVII variants in conditioned medium (1.2 nmol/l). Mock medium was used as negative control. Beads were incubated for 1 h at 37°C with serial dilutions of patient plasma (0.64–0.005 BU/ml). Detection was performed through a goat phycoerythrin-conjugated anti-human IgG (goat PE-IgG, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA).

Isotopic analysis of inhibitory antibodies

rFVIIa-coupled beads (3,000/well) were incubated for 1 h at 37°C with 1/100 diluted patient plasma. Commercial FVII-deficient plasma as well as beads not coupled with rFVIIa were used as negative controls. The detection of total IgG (IgGt) or IgG 1–4 subclasses directed against rFVIIa was performed by using the goat PE-IgG or isotype-specific anti-human IgG (IgG1 to IgG4) antibodies (Beckman Coulter, Fullerton, CA, USA), respectively.

In both assays, beads were resuspended and read on the Bio-Plex™ platform (Bio-Plex 200 Station, Bio-Rad Laboratories). Acquisition software (BioPlex Manager™ 3.0, Bio-Rad Laboratories) was used to analyse data. Results were expressed as Fluorescence Intensity (FI).
Fluorescent functional assays

Functional assays were adapted from the Bethesda-Nijmegen method (23, 27), used to detect coagulation inhibitors in patients. Patient plasma containing inhibitory antibodies was serially diluted in commercial FVII-deficient plasma.

Functional assays with rFVIIa

Diluted patient plasma was incubated (1:1 vol/vol) for 2 h at 37°C with 0.5 nmol/l rFVIIa prepared in dilution buffer (20 mmol/l Heps pH 7.4, 150 mmol/l NaCl, and 0.1% PEG-8000). The mixture was then diluted 1:20 in dilution buffer and sub-sampled in a 96-well microplate. Commercial FVII-deficient and patient plasma alone were used as controls. Coagulation was triggered by the addition (1/100 of the reaction volume) of Innovin (Dade "Innovin", Siemens Healthcare, Marburg, Germany) and the reaction was monitored by fluorogenic substrates for FXa (150 µmol/l, SpectroFluorTM FXa, American Diagnostica Inc.) or thrombin (250 µmol/l, Thrombin Substrate III, EMD Biosciences Inc., La Jolla, CA, USA) prepared in reaction buffer (20 mmol/l Heps pH 7.4, 150 mmol/l NaCl, 5 mmol/l CaCl₂, and 0.1% PEG-8000). The generation of FXa or thrombin was measured as fluorescence emission (Relative Fluorescence Units, RFU; 360 nm excitation, 465 nm emission) over time on a microplate fluorometer (Fluoroskan Ascent FL, Thermo Fisher Scientific, Helsinki, Finland).

FXa generation assays with R462X homozygote plasma

The FXα generation activity in plasma containing the FVII-462X variant was compared with that in a reference plasma containing 2% FVII (2% FVII PNP) prepared by diluting PNP in commercial FVII-deficient plasma. Serially diluted inhibitor-containing plasma was incubated (1:1 vol/vol) for 2 h at 37°C with either 2% FVII PNP or FVII-462X, which were also incubated alone as reference. Commercial FVII-deficient and inhibitor-containing plasma were used as controls. Samples were processed as described above.

FXa generation assays with recombinant FVII variants

Conditioned medium containing rFVII (0.5 nmol/l) was mixed 1:1 with diluted patient plasma and processed as described above. The rFVII variants added 1:1 to commercial FVII-deficient plasma were used as reference, and mock-medium mixed with commercial FVII-deficient or diluted patient plasma as controls.

Data analysis

All assays have been conducted at least in duplicate. Data were analysed by the statistic software GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The parameters of functional assays were extrapolated from the first derivative of relative fluorescence units (RFU) as function of time (minutes). In binding assays, half-maximal binding was obtained by a non-linear regression analysis of the Optical Density or Fluorescence Intensity as function of antibody amount (BU/ml).

Results

The study stems from the identification of high titre anti-FVII inhibitory antibodies (9) developed in a severe FVII-deficient patient after replacement therapy with rFVIIa or pdFVII. The patient is homozygous for the F7 p.A354V-p.P464Hfs mutation, which causes the reading frameshift of the last codons, thus resulting in an elongated FVII variant (►Figure 1). This dysfunctional protein (FVII coagulant activity <1%) with an abnormal carboxy-terminal circulates in plasma at very low levels (FVII antigen 1.2% of normal). Isotypic analysis on patient plasma obtained from different collections over time revealed a large predominance of IgG isotype 1 ranging from 79 to 98% of the total IgG.

Activated FVII molecules are preferentially recognised by antibodies

By ELISA-based assays we investigated the binding of the anti-FVII antibodies, present in patient plasma, to the zymogen or activated FVII forms. The antibodies efficiently bound both rFVIIa and zymogen pdFVII, with higher affinity for rFVIIa, as indicated by their half-maximal binding at 0.54 ± 0.04 and 0.78 ± 0.07 BU/ml, respectively (►Figure 2A).

Binding was further investigated by competition experiments. Coated FVII molecules were incubated with the antibodies alone or with rFVIIa or pdFVII as competitors. The rFVIIa was the best competitor at each concentration tested, particularly when pdFVII was used as the coated molecule (►Figure 2B). Consistently, the activation of pdFVII to pdFVIIa improved its competition efficiency. At the competitor concentration range displaying a virtually linear response (0.125–1 nM), the residual binding of anti-

---

**Figure 1:** Schematic representation of the carboxyl-terminal region of the FVII variants. The primary sequence of the carboxyl-terminal region of the wild-type FVII is shown. The number of deleted residues in the FVII variants is indicated on the right. The arrow above the A354V-P464Hfs variant indicates the frameshifted (dark grey box, residues 464–466) and elongated (+28 aa) regions.
Bodies to coated FVII molecules dropped from 97.0 ± 2.8 % to 43.1 ± 3.9 % (rFVIIa) and from 93.1 ± 0.8 % to 60.8 ± 0.4 (pdFVIIa), while that of zymogen pdFVII was slightly reduced to 88.0 ± 0.7 % (Figure 2B). Taken together, our observations indicated that the antibodies in patient plasma preferentially recognised the activated FVII form.

To further characterise the features of inhibitors toward activated FVII, we performed ELISA-based binding assays with native and reduced rFVIIa (Suppl. Figure 1, available online at www.thrombosis-online.com). The treatment with reducing agents (10 mmol/l β-mercaptoethanol) prevented the recognition by antibodies, thus pointing to the presence of conformational epitopes.

The anti-FVII antibodies impaired the initial phase of coagulation

FXa and thrombin generation assays were set up in commercial FVII-deficient plasma supplemented with rFVIIa (0.5 nmol/l), which mimics the patient treatment. The addition of the inhibitor-containing patient plasma resulted in a remarkable and dose-dependent prolongation of lag times, particularly in FXa generation (Figure 3A, B). In both assays the maximal effects were achieved with inhibitor levels as low as 1 BU/ml, and 8 BU/ml did not produce any further prolongation of lag times, as indicated by the lag time ratios (Figure 3C). In our experimental setting the FXa generation at 8 BU/ml corresponded to that produced by 0.1 nmol/l rFVIIa in the absence of inhibitors, a significant residual activity (~20 %) that indicated a second-order kinetics of inhibition. In all assays, the other parameters of thrombin and FXa generation curves such as the peak and the area under the curve were not appreciably affected (Figure 3). Taken together these observations, and particularly the differential impact on lag times, indicated the presence of inhibitory antibodies affecting the initiation phase of coagulation.

The carboxy-terminal region as candidate target for the anti-FVII antibodies

The inferred structural differences between the small amounts of the elongated variant circulating in patient plasma and the infused FVII molecules led us to hypothesise that the carboxy-terminal of normal FVII/FVIIa is recognised by the anti-FVII antibodies (Figure 1).

To address this issue, we performed functional assays in plasma from a patient homozygous for the p.R462X nonsense change. This mutation causes low levels of circulating FVII molecules lacking the last five residues (FVII-462X) (24).

Plasma from the p.R462X homozygote or PNP, diluted in commercial FVII-deficient plasma to produce a comparable FXa generation activity, were incubated with increasing amounts of inhibitors (Figure 4). Strikingly, the activity in the p.R462X homozygote plasma was poorly affected, as indicated by the almost overlapping FXa generation curves, whereas a strong dose-dependent prolongation of lag times was evident for PNP. Overall these experiments in a plasma model with a naturally truncated FVII variant provided evidence for a role of the carboxy-terminal region as target for the inhibitory antibodies.

Design of inhibitor-resistant FVII variants

Our results prompted us to explore recombinant carboxy-terminal truncated FVII variants resistant to the inhibition. The FVII-462X circulates in plasma of the FVII-deficient patient at very low levels, and its recombinant expression does not produce appreciable amounts of protein, as also observed for the rFVII-463X variant (24), which prevents further analysis and use of these molecules. For these reasons, we expressed and tested the truncated rFVII-466X, rFVII-465X and rFVII-464X variants lacking up to three residues (Figure 1), which were expressed in...
media at appreciable levels and displayed a virtually normal specific activity (Suppl. Figure 2, available online at www.thrombosis-online.com).

To assess whether these truncated variants were able to escape inhibition, their activity was challenged by increasing amounts of inhibitors. Appreciable differences were observed among variants and, noticeably, the rFVII-464X molecule was the least inhibited, as indicated by the slightly prolonged time (Figure 5A). Remarkably, the time prolongation for the rFVII-464X variant at the inhibitor titre of 8 BU/ml (right panel) was similar to that ob-

![Figure 3: The inhibitory antibodies prolong the lag times in FXa and thrombin generation assays. A-B) FXa (A, open circles) and thrombin (B, closed circles) generation activity in FVII-deficient plasma supplemented with 0.5 nmol/l rFVIIa, incubated with increasing amounts of inhibitor-containing patient plasma. The amount of inhibitors (0, 0.25, 1 and 8 BU/ml) is reported on top of the curves. For the sake of clarity, the curves with 0.125 and 0.5 BU/ml, analysed in C, are not shown. RFU, Relative Fluorescence Units. C) Relationship between Lag Time Ratio (lag time with inhibitor/lag time without inhibitor) and the amount of inhibitory antibodies (BU/ml) in FXa (open circles) and thrombin (closed circles) generation assays. Results are expressed as mean ± standard deviation from two independent experiments.](image-url)
served at 0.25 BU/ml (left panel) for the rFVII-wt (wild-type), and corresponded to a 40-time reduction in inhibition.

To corroborate findings obtained in functional assays, we tested the affinity of the anti-FVII antibodies towards the recombinant truncated molecules by binding assays on fluorescent beads. We observed the lowest affinity for the shortest rFVII-464X variant (▶Figure 5B), which displayed an estimated half-maximal binding (0.198 ± 0.003 BU/ml) significantly higher than that of rFVII-wt (0.032 ± 0.002). Intermediate values were observed for the rFVII-465X (0.06 ± 0.004) and rFVII-466X (0.037 ± 0.003) molecules. These results indicated that, among the recombinant truncated variants tested, the rFVII-464X was recognised with the lowest affinity by the antibodies in patient plasma.

Overall data indicated that the shortest rFVII-464X molecule, characterised by appreciable biosynthesis and catalytic activity, was remarkably less recognised by antibodies and resistant to inhibition, which also supports the FVII carboxyl-terminus as a main target for the antibodies.

Discussion

We investigated the intriguing observation of a case of anti-FVII inhibitors developed after replacement therapy in a patient homozygous for the FVII p.A354V-p.P464Hfs mutation, a molecular defect compatible with residual circulating levels of a dysfunctional FVII molecule (12). To characterise the main features of the anti-FVII inhibitors we set up a number of sensitive functional and binding assays in plasma systems with natural and recombinant FVII molecules.

FXa and thrombin generation assays directed to the functional characterisation of the inhibitors clearly indicated their impact on the initiation phase of coagulation. The prolonged lag times, a parameter substantially influenced by FVII activity levels (28–30), are coherent with a FVII-specific inhibition affecting the generation of FXa and, as a consequence, of thrombin. The residual activity was appreciable even at the highest inhibitor amount tested, thus indicating a second-order kinetics of inhibition. Moreover, isotypic analysis revealed in patient plasma a large predominance of IgG isotype 1 that, based on data from HA, appears to correlate with a favourable condition in patients (31, 32).

The clinical history of the patient under study reports several therapeutic treatments with rFVIIa or pdFVII (9). High titres of inhibitors were detected after the administration of consecutive doses of rFVIIa, and subsequently after treatment with pdFVII. In principle, both activated and zymogen FVII molecules could be involved in the immune response. Binding assays indicated that the antibodies recognised both FVII forms, with a higher affinity for rFVIIa. This observation was corroborated by competition assays revealing that pdFVII, once converted into its activated form pdFVIIa, displayed a competition efficiency similar to that of rFVIIa, and indicated that the rFVIIa recombinant features poorly contributed to the recognition.

To help explaining these observations we inspected the crystallographic structures of FVII (1JBU) (33) and FVIIa (2PUQ) (34) in which the carboxyl-terminus is exposed on the catalytic domain and is structurally shaped by a network of hydrogen bonds, particularly with serine 292 (Suppl. Figure 3, available online at www.thrombosis-online.com). Superimposition of the two structures

Figure 4: The naturally-occurring truncated FVII-462X variant is resistant to inhibition. A) Plasma from a patient homozygous for the p.R462X nonsense mutation (FVII-462X variant, upper panel, open circles) and plasma containing 2 % of FVII (2 %FVII PNP, lower panel, closed circles) were tested upon addition of increasing amounts of inhibitors (0–8 BU/ml) and the residual activity was measured. For the sake of clarity the curves with 1 and 4 BU/ml, analysed in B, are not shown. RFU, Relative Fluoresce Units. B) Lag Time Prolongation (difference between lag time values in the presence and in the absence of inhibitors) at increasing amounts of antibodies as in panel A.
Figure 5: The rFVII-464X variant displays reduced binding to antibodies and is resistant to inhibition. A) FXa generation activity of rFVII variants (1.2 nmol/l) in plasma with inhibitory antibodies. The left panel reports the Lag Time Prolongation magnified for low inhibitor amounts (≤0.5 BU/ml). The Lag Time Prolongation for the rFVII-464X variant at 8 BU/ml (right panel) is similar to that observed at 0.25 BU/ml (left panel) for the rFVII-wt, and corresponds to a 40-time reduction in inhibition. B) Binding assays with rFVII variants bound to coated fluorescent beads and exposed to patient antibodies. Beads were coated with a monoclonal antibody recognising the FVII light chain to expose the heavy chain containing the carboxyl-terminus, and favour interaction with antibodies in patient plasma. Results are expressed as mean ± standard deviation from two independent experiments. The reduced inhibition and binding of the shortest rFVII-464X variant is highlighted by the bold dotted line.

revealed differences in the length of hydrogen bonds, which might underlie subtle changes between the putative epitope in the FVII and FVIIa forms potentially supporting the differential binding affinities observed in our assays.

Taken together these results indicated a preferential recognition of activated FVII forms, which indeed may have functional implications, and also beyond the treatment of FVII deficiency. It is of activated FVII forms, which indeed may have functional implications. The reduced inhibition and binding of the shortest rFVII-464X variant is highlighted by the bold dotted line.

acquired haemorrhagic conditions, including haemophilia patients with FVIII inhibitors (37–39).

Previous studies in HA patients with missense mutations who developed inhibitors (18, 19, 40, 41) suggest that, beside other genetic and acquired components (14–17), structural differences between the endogenous variant and the infused therapeutic proteins contribute to the immune response (3, 40). The patient under investigation is homozygous for the p.A354V-p.P464Hfs mutation that causes a reading frameshift of the last residues and of the natural stop codon, thus resulting in an elongated FVII variant (Figure 1). This observation led us to hypothesise that antibody epitopes could reside in the carboxyl-terminal region of infused FVII molecules.

We took advantage of a FVII variant, detected in a FVII-deficient patient homozygous for the p.R462X nonsense mutation (24), which lacks the last five residues of the carboxyl-terminus. Strikingly, FXa generation assays revealed that the activity of the FVII-462X variant, and particularly the lag times, were poorly affected by the inhibitors when compared to pooled normal plasma, thus supporting our hypothesis. Although the FVII-462X protein represents a unique example of a naturally-occurring variant with gain-of-function features able to escape inhibition, this variant is produced at trace levels in eukaryotic cells. As a consequence, a panel of rFVII variants with progressive deletions in the carboxyl-terminus, efficiently secreted and endorsed of normal procoagulant activity, was challenged and, intriguingly, found to display a remarkable resistance to inhibition, inversely related to the extension of the carboxyl-terminal region beyond position 463. Consistently, the shortest variant (rFVII-464X), which underwent the weakest inhibition, displayed the lowest binding affinity for the antibodies, thus pinpointing potential therapeutic features.

We are aware that this study has some limitations. Although the parallel impact on binding and functional inhibition is compatible with antibodies recognising the wild-type carboxyl-terminus and inhibiting FVIIa activity in plasma, their polyclonal features may produce confounding effects, thus not allowing to provide a formal evidence for a dual role exerted by the same antibody species. While the preferential recognition of antibodies for the activated FVII would suggest an impairment of FVII activity rather than activation, our experimental design did not permit the dissection of the biochemical mechanisms underlying the functional inhibition. Moreover, we investigated a very rare case of anti-FVII inhibitors, which does not permit any epidemiological conclusion. It is worth noting that within the STER we have enrolled 12 p.A354V-p.P464Hfs homozygotes but only the patient under study developed anti-FVII inhibitors (9). A similar picture has been observed for the recurrent p.Q160R mutation (6, 12). Altogether these observations, comparable to those in HA, further highlight the complex interplay of intragenic mutations with other genetic and acquired components (14) in shaping the risk of developing inhibitors, which is far from being understood.

In addition, the inhibitors appear to be related to the specific FVII molecular defect. Hence, the inhibitor-resistant rFVII-464X molecule represents the proof-of-principle for a personalised therapeutic approach with limited applicability to other patients.
What is known about this topic?

- Inhibitory antibodies, which represent a frequent and severe complication of replacement therapy of haemophiliacs, are rare and virtually not characterised in FVII deficiency
- Activated recombinant FVII (rFVIIa) is widely used in several bleeding disorders, including HA and HB patients with anti-FVIII and anti-FIX inhibitors.

What does this paper add?

- We report the first characterisation of anti-FVII antibodies, preferentially recognizing activated FVII and related to the F7 gene mutation.
- We report that a natural FVII variant is resistant to inhibition and identify a recombinant, truncated FVII variant poorly recognised and inhibited by the antibodies.

However, this variant might help circumventing immune responses to novel therapeutic fusion proteins modifying the FVII carboxyl-terminus (36).

In conclusion, we report the first characterisation of anti-FVII inhibitory antibodies developed after replacement therapy, and produce experimental evidence for i) higher affinity of antibodies for activated than zymogen FVII molecules, ii) specific inhibition of the initiation phase of coagulation, and iii) lower affinity and inhibition of truncated variants than full-length FVII, thus indicating recognition of the “wild-type” carboxyl-terminus. This genotype-related strategy led us to identify an efficiently secreted and procoagulant molecule (rFVII-464X), poorly recognised and inhibited by antibodies. Taken together our results provide a paradigmatic example both of mutation-related inhibitory antibodies and inhibitor-resistant recombinant molecules, which might represent novel therapeutic tools.

Acknowledgements

This work was supported by AIFA (AIFA 2008 – Bando per le malattie rare – Progetto RF-null-2008–1235892) (Francesco Bernardi, Mirko Pinotti), Telethon-Italy (GGP09183)(Alessio Branchini, Marcello Baroni, Francesco Bernardi, Mirko Pinotti) and CSL Behring (Muriel Giansily-Blaziot and Caroline Pfeiffer).

Conflicts of interest

None declared.

References

14. Hay CR, Oller W, Pepper L, et al. HLA class II profile: a weak determinant of abnormal recognition of the “wild-type” carboxyl-terminus. This genotype-related strategy led us to identify an efficiently secreted and procoagulant molecule (rFVII-464X), poorly recognised and inhibited by antibodies. Taken together our results provide a paradigmatic example both of mutation-related inhibitory antibodies and inhibitor-resistant recombinant molecules, which might represent novel therapeutic tools.

Acknowledgements

This work was supported by AIFA (AIFA 2008 – Bando per le malattie rare – Progetto RF-null-2008–1235892) (Francesco Bernardi, Mirko Pinotti), Telethon-Italy (GGP09183)(Alessio Branchini, Marcello Baroni, Francesco Bernardi, Mirko Pinotti) and CSL Behring (Muriel Giansily-Blaziot and Caroline Pfeiffer).

Conflicts of interest

None declared.

References

14. Hay CR, Oller W, Pepper L, et al. HLA class II profile: a weak determinant of abnormal recognition of the “wild-type” carboxyl-terminus. This genotype-related strategy led us to identify an efficiently secreted and procoagulant molecule (rFVII-464X), poorly recognised and inhibited by antibodies. Taken together our results provide a paradigmatic example both of mutation-related inhibitory antibodies and inhibitor-resistant recombinant molecules, which might represent novel therapeutic tools.

Acknowledgements

This work was supported by AIFA (AIFA 2008 – Bando per le malattie rare – Progetto RF-null-2008–1235892) (Francesco Bernardi, Mirko Pinotti), Telethon-Italy (GGP09183)(Alessio Branchini, Marcello Baroni, Francesco Bernardi, Mirko Pinotti) and CSL Behring (Muriel Giansily-Blaziot and Caroline Pfeiffer).

Conflicts of interest

None declared.

References