Antibodies in the Treatment of Haemophilia A— A Biochemical Perspective

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Abstract

Keywords

- haemophilia
- factor VIII
- antibody

Zusammenfassung

Schlüsselwörter

- Hämophilie
- Faktor VIII
- Antikörper

Replacement therapy has been proven effective in the management of bleedings in haemophilia A. Nevertheless, this approach comes with several shortcomings, like the need for frequent intravenous infusions and the development of neutralizing antibodies in 20 to 30% of the patients with severe haemophilia A replacement. This has led to the development of novel strategies to expand the spectrum of treatment options, some of which are based on antibody technology. These include a bispecific antibody that bridges enzyme factor IXa and substrate factor X, monoclonal antibodies that block the function of tissue factor pathway inhibitor, and a factor VIII–nanobody fusion protein with strongly enhanced von Willebrand factor binding. In this review, functional and mechanistic considerations on the use of these antibody variants will be discussed.

Die Ersatztherapie hat sich bei der Behandlung von Blutungen bei Hämophilie A als wirksam erwiesen. Dennoch bringt dieser Ansatz einige Nachteile mit sich, wie beispielsweise die Notwendigkeit häufiger intravenöser Infusionen und die Entwicklung von neutralisierenden Antikörpern bei 20 bis 30% der Patienten mit schwerer Hämophilie A. Dies hat zur Entwicklung neuer Strategien geführt, um das Spektrum der Behandlungsoptionen zu erweitern, von denen einige auf der Antikörpertechnologie basieren. Dazu gehören ein bispezifischer Antikörper, der den Enzymfaktor IXa und den Substratfaktor X überbrückt, monoklonale Antikörper, die die Funktion des Tissue factor-Pathway-Inhibitors blockieren, und ein Faktor-VIII-Nanobody-Fusionsprotein mit stark verstärktem von Willebrand-Faktor. In dieser Übersichtsarbeit werden funktionale und mechanistische Überlegungen zur Verwendung dieser Antikörpervarianten diskutiert.

Introduction

The activated derivative of factor VIII (FVIIIa) participates in the coagulation cascade as a nonenzymatic cofactor for activated factor IX (FIXa) in the generation of activated factor X (FXa).¹ Its physiological relevance in this enzymatic reaction is illustrated by the notion that the functional absence of FVIII predisposes to a severe bleeding diathesis, known as haemophilia A.

For decades, the treatment of haemophilia A has mainly consisted of replacement therapy using FVIII concentrates of varying purity. As of today highly purified plasma-derived or recombinant FVIII concentrates are broadly used for the prophylactic treatment of haemophilia A, thereby not only markedly reducing spontaneous bleeding events but also enabling home therapy.^{2,3} Although effective, replacement therapy comes with several disadvantages, like the need for frequent

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Emicizumab: bridges FIXa & FX, promoting FX activation by FIXa

Anti-TFPI: blocks TFPI function, reducing inhibition of TF/FVIIa/FXa and prothrombinase complexes by TFPI

FVIII-nanobody fusion protein: Enhanced VWF binding causing prolonged half-life and reduced immunogenicity

Fig. 1 Antibodies in haemophilia treatment. The mechanisms of action of three different types of antibodies that are or may be useful in the management of haemophilia are schematically depicted.

intravenous applications and the development of anti-FVIII antibodies in 20 to 30% of the patients with severe haemophilia A.⁴⁻⁶ It is not surprising therefore that a search for alternative strategies to improve haemophilia A treatment has been ongoing. In this review, we will focus on antibody-based treatment options, and three examples will be discussed (→ Fig. 1). First, a bispecific antibody (emicizumab) that combines FIXa and FX to enhance FXa generation; second, antibodies that block the function of tissue factor pathway inhibitor (TFPI), thereby promoting activity of the tissue factor/factor VIIa (TF/FVIIa) and prothrombinase complex; and third, a FVIII-nanobody fusion protein that displays prolonged survival and reduced immunogenicity.⁷⁻⁹ Importantly, this review will limit itself to the functional and mechanistic aspects of the antibodies, and will not discuss clinical efficacy.

Bispecific Antibody Emicizumab: Comparison with FVIII

Emicizumab (also known as ACE910 or Hemlibra) is a bispecific antibody that interacts with FIX/FIXa and FX/FXa.⁸ The rationale behind this antibody is that it brings the enzyme FIXa and its substrate FX in close proximity, favouring conversion of FX into FXa. To some extent, this antibody resembles FVIIIa, the cofactor within the tenase complex that is also capable of simultaneously interacting with FIXa and FX. Nevertheless, emicizumab and FVIII are very different proteins in terms of structure and regulation, and consequently they will act differently during coagulation. To understand these differences, it is perhaps convenient to first provide a reminder of how FVIII actually functions. We have recently described the mode of action of FVIII in great detail,¹⁰ and will therefore only provide a summary of the key points in this regard.

On/off switch: FVIII circulates as a precofactor that requires activation by thrombin and/or FXa before it becomes an active cofactor. However, because FVIIIa is a very labile protein, it will lose its function by just falling apart within minutes following activation.¹¹ Also, FVIIIa is an

efficient substrate for activated protein C, which is able to inactivate FVIIIa via site-specific proteolysis.¹² FVIIIa activity is thus regulated by an on/off switch.

Specific cofactor functions: FVIIIa's cofactor function consists of different explicit tasks. FVIIIa initiates formation of the tenase complex by recruiting FIXa to the phospholipid surface, limiting the movement of the proteins to a two-dimensional space.¹³ While interacting with FIXa, FVIIIa stabilizes the protease domain of FIXa.¹⁴ This interaction has two main functions: to reduce the flexible movements of the surface loops that surround the active centre and to position the active centre at the appropriate height from the phospholipid surface. Both functions are crucial to optimize the catalytic capacity of FIXa. Once in place, the complex is ready to receive FX. By interacting with FVIIIa, the activation peptide of FX is optimally aligned to the active site of FIXa, which further promotes FXa generation.¹⁵ Thus, multiple aspects of FVIIIa cofactor function define its enhancing potential within the tenase complex.

Specificity: Although FIXa binds equally well to FVIII or FVIIIa (with this interaction only being limited by the presence of von Willebrand factor [VWF]), the opposite is untrue. FVIIIa (or FVIII) is unable to interact with the FIX zymogen.¹⁶ This restricts the formation of the complex to one between the enzyme and cofactor, rather than between the zymogen and cofactor. Furthermore, FVIIIa is able to interact with FX, while less so with FXa.¹⁵ Indeed, the low affinity for FXa compared with FX allows the release of FXa from the FIXa/FVIIIa complex, making the tenase complex available for a next substrate molecule. Hence, FVIIIa displays specificity toward both the enzyme and substrate.

Stimulating activity: The magnitude by which FVIIIa enhances the catalytic activity of FIXa is often determined in assays using purified components. Accordingly, variables like phospholipid concentration and composition, as well as concentrations of the proteins, are determinant for the catalytic efficiency that is measured. In general, FVIIIa enhances FIXa activity by 1,000- to 1 million-fold.

Limiting factor within the tenase complex: To establish what is the limiting factor within the tenase complex, it is of

relevance to consider the respective protein concentrations. It is obvious that FVIII concentrations (0.3–1 nM) are considerably lower than those of FIX (90 nM) or FX (135 nM). The actual concentrations of FVIIIa and FIXa during in vivo coagulation are difficult to establish, but even if we consider that 50% of FVIII is activated versus 5% of FIX, this would still mean 9 to 30-fold less FVIIIa than FIXa (0.15–0.5 vs. 4.5 nM). It is thus fair to assume that FVIIIa is the limiting factor within the tenase complex.

In view of this complex mechanism of action and regulation of FVIIIa, it is obvious that the characteristics of the antibody-based protein emicizumab will be rather different. A first important difference is that emicizumab has similar affinity for FIX versus FIXa and for FX versus FXa.¹⁷ This indicates that the specificity of FVIIIa toward FIXa and FX is no longer present in the bispecific antibody. It is therefore possible that the following ternary complexes will be formed in the circulation: emicizumab/FIX/FX, emicizumab/FIXa/FX (which is the one that is relevant for FXa generation), emicizumab/FIX/FXa, and emicizumab/FIXa/FXa. How much of each complex is being formed is very much dependent on the respective concentrations of the individual constituents. Based on concentrations and respective affinities, it is possible to calculate how much of each complex is formed. In the normal circulation and at therapeutic emicizumab concentration of 55 µg/mL (370 nM), the emicizumab/FIX/FX complex will be the most abundant, with ~0.8 nM ternary complex.¹⁷

Interestingly, in normal individuals (and there is no reason to assume that this will be different in haemophilia A patients), there is ~120 pM of FIXa that is constitutively present in the circulation.¹⁸ Since emicizumab does not make any distinction between FIX and FIXa, only a small portion of these 120 pM of FIXa will bind to emicizumab. Indeed, the ternary complex relevant for FXa generation (emicizumab/FIXa/FX) present under basal conditions in these treated patients can be calculated to be ~2 pM. Of note, since emicizumab is not regulated by an on/off switch, this may indicate that there is a constitutive background activity of this emicizumab/FIXa/FX complex. It is not to be excluded that such low background activity may act already during small subclinical bleeds that do not provoke a full trigger of the coagulation cascade.

An important question is of course how efficiently does emicizumab enhance FIXa activity in comparison to FVIIIa? The antibody configuration of emicizumab will prevent it from capturing FIXa and FX to the phospholipid surface (something that does not exclude the activation reaction to be still phospholipid-dependent). Furthermore, with the emicizumab binding site being located in the epidermal growth factor 1 (EGF1)-like domain of FIXa, the bispecific antibody is also unlikely to modify the flexibility and position of the FIXa protease domain to a significant extent. It is therefore difficult to expect that emicizumab to be as efficient as FVIIIa in stimulating FX activation. Experiments using purified components showed that emicizumab was approximately 10-fold less efficient in enhancing the catalytic efficiency by which FIXa activates FX.^{8,17}

Emicizumab: Monitoring Using Activity Assays

To monitor emicizumab-based therapy in the patients, two issues are of relevance. First, one might want to verify levels of emicizumab during therapy. In the current clinical trials, a specific enzyme-linked immunosorbent assay (ELISA) system using anti-idiotypic antibodies has been applied.¹⁹ Alternatively, one could also employ a chromogenic FVIII activity assay, with the necessity of such an assay to contain human FIXa and FX (for example Biophen FVIII:C by Hyphen Biomed), which uses emicizumab as standard. The main difficulty is to find a so-called FVIII-equivalence using activity assays. Several assays are available to measure FVIII activity, like the activated partial thromboplastin time (aPTT), thrombin generation assays (initiated by TF or factor XIa), or clot waveform analysis. Each of these assays is based on FVIII being the limiting factor, while the amount of FIXa that is generated in these tests is quite variable from one test to the other, and even depends on the activating reagent used. Knowing that emicizumab is present in large excess over all other relevant components (FIXa and FX) and is always in its “on”-mode, it will thus be the amount of FIXa present that will determine the activity of emicizumab. With the FIXa concentrations being different from one assay to the other, it will be complicated to assign a FVIII-like value to a certain emicizumab concentration. Care should therefore be taken to extrapolate findings concerning emicizumab in laboratory assays to the in vivo haemostatic potential of this molecule. We would like to emphasize that this does not mean that emicizumab activity in vivo is *per definition* lower than what will be measured in laboratory assays. It cannot be excluded that emicizumab has the intrinsic capacity to modulate the extent of FIXa formation in a way that goes unnoticed in the regular activity assays. For example, it is known that FXa is able to generate the FIX activation intermediate FIXα, which is a much better substrate than FIX for the TF/FVIIa complex. Akin to emicizumab promoting formation of FXa by FIX, it is theoretically possible that it also promotes FXa-mediated FIXα formation, which in the presence of TF/FVIIa is then readily converted into FIXa. This pathway could eventually explain why emicizumab seems to have higher activity in TF-based thrombin generation assays compared with FXIa-based thrombin generation assays.

Emicizumab in Combination with Other Procoagulant Agents

Under certain conditions, emicizumab has been or will be used in combination with other procoagulant proteins, such as FVIII concentrates or FVIII-bypassing agents like factor eight inhibitor bypassing activity (FEIBA) or FVIIa. It is of interest to consider how the co-use of such products impacts coagulation. In case of FEIBA use, several issues need to be considered. First, FEIBA consists of many different proteins, including high concentrations of prothrombin, FX, and FIX.²⁰ By adding FIX and FX to the circulation, one automatically increases levels of the ternary complexes. A dose of 100 U/kg

of FEIBA will more than double the concentration of the ternary emicizumab/FIX/FX complex in the circulation, assuming a therapeutic emicizumab level of 370 nM.¹⁷ Furthermore, FEIBA contains considerable amounts of FIXa and a dose of 100 U/kg may ultimately result in circulating FIXa levels that may reach 1 nM.^{21,22} Since emicizumab makes no distinction between FIX and FIXa, and does not require an on-switch to become active, the presence of FIXa may directly be used to trigger FXa generation: the ternary, FXa-generating emicizumab/FIXa/FX complex may rise to a concentration of 20 pM.

With regard to the use of FVIIa, it will be rather different. The use of FVIIa will not change the basal FIX or FX levels, suggesting ternary complex formation remains unaltered. However, FVIIa will cause the generation of both FIXa and FXa, thereby increasing the participation of these proteins in the complexes with emicizumab. Primate studies using a dose of 50 µg/kg FVIIa revealed that such a dose doubles the FIXa concentration in the circulation from ~120 to 240 pM.²³ This suggests that that FVIIa may contribute to an increase in active emicizumab/FIXa/FX complexes, albeit probably to a lesser extent compared with FEIBA.

As for the combination with FVIII, two distinct situations should be considered. First, in patients having no anti-FVIII antibodies receiving regular doses of FVIII concentrates (25–50 U/kg), FVIII will be present together with emicizumab and compete for the interaction with FIXa. Since FVIIIa displays much higher affinity for FIXa than emicizumab (2–15 nM vs. 1.5 µM), and will not be diluted to nonactivated FIX zymogen, it seems likely that any FIXa present will first choose to partner with FVIIIa. However, also in these situations, FIXa will be present in excess over FVIIIa. The remaining FIXa thus remains available to interact with emicizumab. Depending on the excess of FIXa, there might therefore be the formation of ternary complexes of emicizumab/FIXa/FX in addition to the tenase complex. However, these ternary complexes are 11-fold less efficient than the tenase complex in generating FXa. Will this be different under conditions where patients receive immune-tolerance therapy? Such patients have inhibitory antibodies against FVIII, and receive high doses of FVIII on a daily basis. Depending on the nature of the inhibitory antibodies and their concentrations, they may prevent FVIII from interacting with FIXa. As such, FIXa remains available for binding to emicizumab. Given the polyclonal character of the antibodies in most patients, it is probable that FVIII will not be interacting with FIXa in these inhibitor patients. Emicizumab will therefore be able to assist FIXa during immune tolerance therapy.

Monoclonal Antibodies Targeting TFPI

TFPI is a Kunitz-type inhibitor, known to interfere with the initial phase of coagulation.^{24,25} Two isoforms are present in humans: TFPIα, which contains three Kunitz domains and a basic C-terminal region, and TFPIβ in which the third Kunitz domain and the basic region are replaced by a glycosylphosphatidylinositol-binding sequence. The mode of action of TFPI is perhaps less straightforward than many seem to

appreciate.^{24,26} At low concentrations, its Kunitz-2 domain interacts loosely with FXa, before isomerizing into a tight complex. This complex is then able to rapidly inhibit the activity of the TF/FVIIa complex, via interactions between the Kunitz-1 domain of TFPI and the FVIIa active site. TFPI alone is a less effective inhibitor of TF/FVIIa compared with FXa/TFPI.

The fact that TFPI plays an important role in the regulation of the initial phase of coagulation has sparked interest related to its use as a target in the treatment of haemophilia.⁷ The rationale would be that by blocking TFPI, more FXa and FIXa will be generated by the FVIIa/TF complex, which in turn will lead to increased thrombin generation and subsequent fibrin deposition and platelet activation. Preliminary studies indeed revealed that anti-TFPI antibodies shorten the coagulation time of haemophilic plasma and reduce the bleeding tendency in a rabbit model of acquired haemophilia A.^{27,28}

As to our knowledge, three monoclonal antibodies targeting TFPI are currently under clinical development: PF-06741086 by Pfizer, BAY1093884 by Bayer Healthcare, and concizumab by Novo Nordisk. Results from efficacy studies using these antibodies are awaited with great anticipation. Meanwhile, it is of interest to reflect on the various aspects of how such monoclonal antibodies affect TFPI biology. Several issues seem to be of relevance in this regard. First, TFPI is not only an inhibitor of the FXa/FVIIa/TF complex, but also recognized as a direct inhibitor of the prothrombinase complex.²⁹ At present, it is unclear which of the three clinical candidates is able to interfere with TFPI-mediated inhibition of the prothrombinase complex, and how this affects the prohaemostatic potential of such antibody.

The second issue relates to the biodistribution of TFPI. The majority of the TFPI molecules are located at the surface and within endothelial cells, while only a small percentage of TFPI is present in plasma, mostly associated to lipoproteins.³⁰ There is also a portion that is stored in platelets.³¹ It will thus be difficult to monitor how much of the total TFPI population will be inhibited by the therapeutic antibodies, while having only access to the patients' plasma. Moreover, in vivo studies using mice with a combined tissue-specific deficiency of TFPI and full deficiency of FVIII revealed that particularly platelet-stored TFPIα is of particular relevance to compensate for the absence of FVIII.³² At this point, it is uncertain which of the three monoclonal antibodies in clinical development is able to specifically target platelet-stored TFPI. Lastly, we know that the FVIIa/TF complex may exert functions beyond haemostasis, in part via protease activated receptor 2 (PAR-2) mediated signalling processes.³³ For example, animal studies have revealed that cell-bound TFPI contributes to reduce tumour metastasis and atherosclerosis.^{34,35} Furthermore, TFPI may also inhibit other proteases that are not involved in haemostasis, and the FVII-activating protease has been reported to be among them.³⁶ It would be important therefore to investigate how the prophylactic use of anti-TFPI antibodies modulates the extra-haemostatic activities of the TF/FVIIa complex and other proteases.

A Factor VIII–Nanobody Fusion Protein

For our third example of how antibodies could be used in the development of novel strategies in the treatment of haemophilia, we would like to focus on a specific kind of antibodies, namely heavy chain-only antibodies.³⁷ Such antibodies are found in members of the Camelid family, which include camels and llamas. These heavy chain-only antibodies lack the light chain that is present in classic immunoglobulin G (IgG) molecules, and consequently all the antigen-binding information is constrained within the variable heavy-chain region of the heavy chain-only antibodies. This variable region (also known as VHH or nanobody) can thus be used similar to the single-chain Fv fragments of classic IgGs. In isolated form, nanobodies have a molecular weight of ~15 kDa. Despite their small size, they can still display high affinity for their target antigen. In the present example of this review, nanobodies have been used as a fusion partner to FVIII.⁹ Of course, many other possibilities to use these specific antibody fragments do exist (see for instance reviews in Beghein and Gettemans 2017, Manglik et al 2017, and Vincke and Muyldermans 2012).^{38–40}

The rationale behind the use of nanobodies as a fusion partner for FVIII lies in the observation that although FVIII binds with high affinity to VWF, there is still 2 to 5% of FVIII that circulates as free protein.^{41,42} Free FVIII differs from VWF-bound FVIII in that it is cleared 6- to 10-fold more rapidly. Moreover, VWF modulates the anti-FVIII immune response by affecting the pathway by which FVIII is endocytosed by antigen-presenting cells.^{43,44} The underlying hypothesis in the design of the FVIII–nanobody fusion protein was therefore to create a FVIII molecule that forms a tighter complex with VWF, thereby reducing the amount of free FVIII. To do so, nanobodies were generated against the VWF D'-D3 domain, the region that contains the FVIII-binding site. These nanobodies indeed display a ~50- to 100-fold slower dissociation rate constant from VWF compared with FVIII.⁹

Two copies of one of these nanobodies, i.e., KB-VWF-013, were cloned into the FVIII complementary DNA (cDNA), replacing the nonfunctional FVIII B-domain.⁹ The final protein, designated FVIII-KB013bv, was produced in mammalian cell lines as a single chain protein, and analysed for function in *in vitro* assays. In terms of activity, FVIII-KB013bv was having full cofactor activity in one stage and chromogenic assays. FVIII-KB013bv further had a 25-fold higher apparent affinity for VWF compared with wild-type FVIII (13 vs. 330 pM), confirming that the presence of the nanobodies considerably improves binding to VWF. This increased affinity means that at a concentration of 1 U/ml, more than 99.8% of FVIII-KB013 is bound to VWF, whereas this is ~96.7% for wild-type FVIII.⁹

The effect of improved VWF binding was evaluated in FVIII-deficient mice. When determining circulatory survival, it was observed that FVIII-KB013bv was cleared 2-fold slower than wild-type FVIII. The increased survival was further established by its capacity to correct bleeding in FVIII-deficient mice 24 hours after a single intravenous injection, whereas by using the same dose wild-type FVIII was no longer able to do so.⁹ Thus, the FVIII–nanobody fusion

protein is fully functional *in vivo* and has a prolonged half-life. With regard to the development of anti-FVIII antibodies, we observed that FVIII-KB013bv was less immunogenic than wild-type FVIII (15% of mice developed antibodies against FVIII-KB013bv vs. 85% developed antibodies against FVIII).⁹ The reason for this difference will require further studies, but may be related to the fact that upon binding of the classic FVIII/VWF complex to antigen-presenting cells, FVIII enters into these cells, whereas VWF remains mainly at the cell surface.⁴⁵ By having a FVIII molecule that sticks to VWF much more tightly, it is possible that less FVIII is able to enter the antigen-presenting cells, resulting in the formation of fewer anti-FVIII antibodies. In comparison to emicizumab and the anti-TFPI antibodies, FVIII-KB013bv is not yet ready to enter clinical evaluation. Nevertheless, it represents an interesting example of how antibodies can be used to optimize treatment in general and that of haemophilia A in particular.

Conclusion

This review has presented three different ways of how antibodies signify novel strategies to innovate the management of bleeding episodes in haemophilia. Importantly, the use of antibodies is not limited to a simple inhibition of the function of its target. Two of the three examples presented here, such as emicizumab that has procoagulant cofactor activity and the FVIII–nanobody fusion protein that ameliorates the molecular properties of the complex FVIII molecule, represent these options. The future will teach us if other types of application will become available to further improve the clinical management of haemophilia.

Disclosures

S.F. has no relevant conflict of interest. P.J.L. has received honorarium/speakers' fee from Bayer Healthcare, CSL Behring, Grifols, Kedrion, LFB, Novo Nordisk, Octapharma, Roche, Spark Therapeutics, Shire, and Sobi. He is also coinventor on patent applications related to treatment options for bleeding disorders, including a patent application describing the FVIII–nanobody fusion protein. P.J.L. is cofounder/coowner of Laelaps Therapeutics.

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