Selection and characterisation of FVIII-specific single chain variable fragments

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Summary
The development of inhibitory anti-FVIII antibodies is currently the most severe complication in the treatment of haemophilia A patients. Inhibitor eradication can be achieved by immune tolerance induction (ITI). Recent findings suggest a correlation between the FVIII-specific IgG subclass distribution and the duration or outcome of ITI. To quantify FVIII-specific IgG subclasses in patients’ plasma FVIII-specific IgG standards are required. Here, the isolation of FVIII-specific single chain variable fragments (scFvs) from synthetic phage display libraries and the characterisation of their FVIII domain specificity are described. The isolated scFv 1G10, which binds to the FVIII A2 domain, was cloned into the context of the four human IgG (hIgG) subclasses and expressed in mammalian cells. Purified 1G10-hIgG1, -hIgG2, -hIgG3 and -hIgG4 are used as standards to determine the absolute amounts and relative contribution of the different FVIII-specific IgG subclasses in future studies. The results from these studies will eventually add to understanding the role of the FVIII-specific IgG subclass distribution as prognostic factor for the outcome of ITI.

Blood coagulation factor VIII (FVIII) is an important component of the blood coagulation cascade. In its processed form FVIII is composed of the A3-C1-C2 light chain (LC) and the heavy chain (HC) linked by a metal bridge, including the domains and A1-A2-B, respectively (13).

Following activation, FVIIIa and FIXa form the tenase complex that activates FX, eventually leading to thrombin burst (5). Mutations in the FVIII gene lead to the absence of FVIII in plasma or to a non-functional protein causing the coagulation disorder haemophilia A (10). Patients with haemophilia A suffer from frequent bleeds, including muscle bleeds, joint bleeds as well as life threatening haemorrhages (9). Therefore haemostasis in patients needs to be restored by the substitution of FVIII concentrates (14).

Up to 30% of patients with severe haemophilia A develop inhibitory anti-FVIII antibodies (FVIII inhibitors) (3). The frequent administration of high doses of FVIII, so-called immune tolerance induction (ITI), can result in the elimination of FVIII inhibitors (2). Different factors influence the outcome of ITI (15) such as the historical peak inhibitor titer, the inhibitor titer at start of ITI and the peak titer during ITI. Furthermore, first results in a case series indicated that successfully treated patients had a higher contribution of FVIII-specific IgG1 whereas the proportion of FVIII-specific IgG4 was increased in patients who failed ITI (18). Interestingly, development of FVIII-specific IgG4 seems to be partly influenced by treatment-related factors (18).

As the formation of anti-FVIII antibodies is associated with increased morbidity, mortality and high cost, it is of great importance to understand the factors in-

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fluencing the outcome of ITI in order to improve overall outcome.

In this study, isolated single chain variable fragments (scFvs) specifically binding to FVIII were isolated from phage display libraries by affinity selection and further characterised. The FVIII domain specificity of scFvs was analysed. ScFv 1G10 was converted into human IgG (hlgG) of the four subclasses.

Material and methods

FVIII biotinylation

Recombinant full length FVIII (FLrFVIII, Kogenate® FS, Bayer, Leverkusen) and B-domain deleted FVIII (BDDrFVIII, Refacto AF®, Pfizer, Berlin, Germany) were biotinylated with the EZ-Link® Sulfo-NHS-LC-Biotinylatation Kit (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer’s instructions. Briefly, 125 µg FVIII (0.5 µg/ml) were dialyzed against 150 mmol/l NaCl, 20 mmol/l HEPES, 5 mmol/l CaCl<sub>2</sub>, 0.01% Tween, pH 7.4 and incubated 1 h with a 50 fold molar excess of Sulfo-NHS-LC-Biotin. Unbound biotin was removed by a second dialysis against the HEPES buffer as described.

Affinity selection by phage display

Biotinylated FVIII was immobilised on Dynabeads® MyOne™ Streptavidin T1 (Invitrogen, Darmstadt, Germany), and the immobilised protein was screened against 1 × 10<sup>12</sup> phages of the Tomlinson I or J libraries (7) (Source BioScience, Nottingham, England) for 2 h. Beads were washed and bound phages were eluted with glycine-HCl, pH 2.2. Eluted phages were incubated with trypsin (1 mg/ml) in 50 mmol/l Tris-HCl, 1 mmol/l CaCl<sub>2</sub>, pH 7.4 for 10 min to cleave the scFv proteins from the phage pIII protein for an unhindered infection of E. coli strain TG1 (Source BioScience).

Library plasmids (phagemids) were amplified and packaged with KM13 helper phage (Source BioScience). Three rounds of selection were performed, and the resulting phage pool was titrated for the analysis of individual clones.

ELISA

FVIII ELISA

ELISA plates were coated with 0.5–1.0 IU/well (~1.1–2.2 µg/ml) FLrFVIII or BDDrFVIII in PBS (Lonza, Walkersville, USA) overnight at 4°C. To test binding to FVIII domains, plates were coated with 0.1 µg FLrFVIII and FVIII LC, 0.2 µg FVIII HC and A2 domain as well as 0.65 µg C2 domain in 20 mmol/l bicine, 2 mmol/l CaCl<sub>2</sub>, pH 9.0. Plates were blocked for 2 h with 5% milk powder, 0.05% Tween-20 in PBS (MPBST). 1 × 10<sup>10</sup> phages or different concentrations of scFv-hlgG were added in MPBST for 2 h at room temperature or at 4°C overnight. Bound phages or scFv-hlgG were detected by incubation with HRP-conjugated anti-M13 antibody (GE Healthcare Amersham, Munich, Germany) 1:5000 or HRP-conjugated goat-anti-human antibody (Caltag H10307, Buc- kingham, Great Britain) 1:1000, respectively, in MPBST for 2 h. Wells were developed with o-phenylenediamine and absorption was measured at 492 nm and 620 nm (reference). Blocked wells without the addition of FVIII were used as a negative control. To analyse binding to denatured FVIII the protein was incubated at 56°C for 30 min before immobilisation.

Competition ELISA

ELISA plates were coated with 0.25 IU/well (~0.56 µg/ml) FVIII in PBS overnight at 4°C, and then blocked for 2 h with MPBST. 1 × 10<sup>10</sup> phages in 50 µl MPBST were preincubated for 30 min, followed by 0.28 µg of murine monoclonal anti-FVIII antibodies GMA012, GMA0815, GMA0817 and GMA0821 (Green Mountain Antibodies, Burlington, USA), ESH8 (American Diagnostics, Stamford, USA) and 1D4 in 50 µl MPBST for 2 h. Bound phages were detected as described for the FVIII ELISA.

Cloning of scFvs into an IgG frame

The scFv sequence of 1G10 contained an amber stop codon UAG that was translated into human IgG (hlgG) of the four subclasses.

Expression of scFv-hlgG proteins and FVIII A2 and C2 domain

The human embryonic kidney cell line 293T (HEK-293T, American Type Culture Collection) was used for the expression of scFv-hlgGs and the FVIII domains A2 and C2 (12). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Sigma-Aldrich, Munich, Germany), 4 mmol/l L-glutamine (Sigma-Aldrich), 100 units/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich) and transiently transfected with the respective expression vectors using polyethyleneimine (Polysciences GmbH, Eppelheim, Germany). 24 h post transfection the medium was exchanged to DMEM supplemented with 4 mmol/l L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 10 µg/ml insulin-transferrin-sodium selenite (Roche Diagnostics GmbH, Mannheim, Germany). Cell culture supernatants were collected for the following three days and stored at −80°C.

Purification of proteins

Purification of recombinant proteins

Supernatants containing scFv-hlgG proteins were adjusted to 10 mmol/l sodium phosphate, pH 7.0. Samples were loaded onto HiTrap Protein A HP columns or for 1G10-hlgG3 Protein G HP columns (GE Healthcare Amersham, Munich, Germany) following filtration. Columns were washed with 20 mmol/l sodium phosphate, pH 7.0...
and proteins eluted with 100 mmol/l citrate, pH 3.0 for protein A or 100 mmol/l glycine-HCl, pH 2.7 for protein G. Eluates were neutralised with 1 mol/l Tris-HCl, pH 9.0 combined, and subsequently concentrated and rebuffered to PBS with a Vivaspin® 20 centrifugal concentrator MWCO 30000 (Vivaproducts, Littleton, USA). Protein concentration was calculated with the Beer-Lambert equation following absorption measurements at 280 nm and fractions were analysed by reducing SDS-polyacrylamide gel electrophoresis followed by Coomassie staining with GelCode™ Blue Stain Reagent (Thermo Fisher Scientific).

Supernatant containing histidine-tagged FVIII A2 or C2 domain was diluted in 10x buffer (500 mmol/l NaHPO₄, 500 mmol/l Na₂HPO₄, 1.5 mol/l NaCl, 100 mmol/l imidazole, pH 8.0) and loaded onto a HisTrap HP column (GE Healthcare Amersham) equilibrated in 50 mmol/l NaHPO₄, 50 mmol/l Na₂HPO₄, 300 mmol/l NaCl, 10 mmol/l imidazole, pH 8.0. Bound FVIII domain was eluted with 50 mmol/l NaHPO₄, 50 mmol/l Na₂HPO₄, 300 mmol/l NaCl, 250 mmol/l imidazole, pH 8.0. Eluates were combined and dialysed against PBS.

Purification of FVIII chains

FLrFVIII was dissociated into HC and LC and polypeptide chains were purified by ion exchange chromatography as described by Healey et al. (8). Briefly, FLrFVIII was dialysed against 20 mmol/l MES, 0.01% Tween-20, 600 mmol/l NaCl, 60 mmol/l EDTA, pH 6.0). The preparation was dialysed with 20 mmol/l MES, 0.01% Tween-20, pH 6.0 to a final concentration of 10 mmol/l EDTA, 100 mmol/l NaCl and loaded onto a HiTrap SP FF column (GE Healthcare Amersham) equilibrated in 20 mmol/l MES, 0.01% Tween-20, 100 mmol/l CaCl, 10 mmol/l EDTA, pH 6.0.

LC bound to the column and was eluted with a linear NaCl gradient (100 mmol/l to 1 mol/l) in 20 mmol/l MES, 0.01% Tween-20, pH 6.0. The flow through containing HC was adjusted to pH 7.2 with 1 mol/l Tris-HCl, pH 8.5 and loaded onto a HiTrap Q FF column (GE Healthcare Amersham, Munich, Germany) equilibrated in 20 mmol/l HEPES, 0.01% Tween-20, 150 mmol/l NaCl, pH 7.2 (buffer G). HC was eluted with a linear NaCl gradient (150 mmol/l to 1 mol/l) in HEPES, 0.01% Tween-20, pH 7.2. Purified HC and LC were dialysed against buffer G.

Bethesda assay

Residual activity of FVIII in the presence of FVIII inhibitors was determined in Bethesda assay as described (13). Briefly, different dilutions of 1G10-hlgG1 were mixed with equal volumes of pooled normal plasma and incubated for 2 h at 37°C. FVIII activity was assessed on a BCT™ (Siemens, Eschborn, Germany) in a one-stage clotting assay. Data was non-linearly fitted with a four-parameter dose-response curve using the program Prism (GraphPad software). The concentration of 1G10-hlgG1 that produced 50% inhibition was used to calculate the specific inhibitory potency (BU/mg).

Results, discussion

Selection and characterisation of FVIII-specific scFvs

Selection of FVIII-specific scFvs from Tolminson I or J libraries was performed against FLrFVIII or BDDrFVIII. The B domain of FVIII, including 907 amino acids, is the largest domain of the FVIII molecule (6). As this could favour the selection of B domain specific scFvs, selection was also performed with BDDrFVIII to increase the chance for isolation of scFvs binding to a functional domain of FVIII.

Both FLrFVIII and BDDrFVIII were biotinylated prior to the affinity selection (data not shown). Selection against biotinylated FLrFVIII and BDDrFVIII resulted in the isolation of two FLrFVIII-specific and three BDDrFVIII-specific scFvs. This set of five FVIII-specific scFvs was analysed for binding to FLrFVIII and BDDrFVIII to elucidate potential B domain-specificity (Fig. 1a). ScFvs 1G3 and 1A7 showed strong binding to both FVIII molecules, which excludes B domain specificity for either of these scFvs. In contrast, scFvs 1B6 and 1C6 only bound to FLrFVIII. Hence, these two phage clones presumably recognise the B domain of FVIII. However, differences in binding to FLrFVIII and BDDrFVIII could be also due to conformational changes in the tertiary structure of the B domain-deleted protein. Binding of scFv 1G10 to FLrFVIII was stronger in comparison to BDDrFVIII, possibly due to different conformational presentation. As binding to BDDrFVIII was still significant this did not imply B domain specificity.

To further analyse the domain specificity of these phage clones, binding to FLrFVIII, HC (A1-A2-B) and LC (A3-C1-C2) as well as to the FVIII A2 and C2 domain was compared in ELISA (Fig. 1a). B domain specificity of 1B6 was further underlined as this phage only bound to FLrFVIII and HC. Phage clone 1C6 bound to FLrFVIII and HC; however, binding to LC was also observed (Fig. 1b). This binding pattern was unexpected due to the lack of binding to BDDrFVIII, which indicated B domain specificity of this clone (Fig. 1a). Alternatively phage binding to LC might be due to the exposure of regions in the LC usually not surface exposed in the B domain deleted FVIII with similarities to the B domain epitope. Binding of clone 1C6 to LC cannot be explained by contamination of the LC fraction with HC protein, as monoclonal HC-specific anti-FVIII antibodies did not show any binding to the LC preparation (data not shown). Based on these inconclusive results domain specificity of 1C6 cannot be clearly specified.

Among the phage clones 1G10, 1G3 and 1A7 that bound to both FLrFVIII and BDDrFVIII domain mapping was only conclusive for 1G10. More detailed, 1G10 bound to FLrFVIII, HC, and A2 domain, specifying this clone as anti-A2 scFv. Phage clone 1G3 only recognised FLrFVIII and 1A7 showed strong binding to LC but not to the individual C2 domain. Hence, phage clone 1A7 presumably binds to amino acids within the A3 and/or C1 domain.

To further confirm the epitope specificity of phage clone 1G10, competition of 1G10 with different monoclonal antibodies (mAbs), for which the epitope is known was tested (Fig. 2a). As expected phage clone 1G10 showed strong binding to FVIII without competing mAb or in the presence of the well-characterised C2-specific ESH8 mAb used as negative control.© Schattauer 2013 Hämostaseologie 4a/2013

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Fig. 1  FVIII domain-specific scFvs analysed by ELISA

a) Identification of FVIII B domain-specific scFvs: FLrFVIII and BDDrFVIII were immobilised on ELISA plates and binding of the indicated phage clones was analysed. Error bars represent standard deviations (SD) of the mean of triplicates. Binding of phage clones 1B6 and 1C6 was dependent on the B domain of FVIII.

b) ScFv 1G10 specifically recognises the FVIII A2 domain: FLrFVIII, heavy (HC) and light chain (LC) as well as FVIII A2 and C2 domain were coated on ELISA plates. Binding of the indicated phage clones was analysed. Error bars represent the SD from mean absorption of duplicates.

Fig. 2  ScFv 1G10 competes with A2-specific antibodies for binding to FVIII and does not bind to denatured FVIII. (Error bars represent the SD of the mean of triplicates.)

a) Binding of 1C6 and 1G10 to FVIII was analysed in the presence of the C2-specific ESH8 mAb (negative control), 1C6-IgG (positive control) and several A2-specific mAbs 012, 1D4, 8015, 8017 and 8021 (for further details of these mAbs see Material + Methods). The absorption in the absence of mAbs was set to 100%.

b) Binding of phages 1C6 and 1G10 to denatured FVIII (FVIII 56°C) and native FVIII (FVIII RT) was analysed by ELISA.
As positive control for competition binding of phage 1C6 was analysed in the presence of 1C6 in the IgG context (1C6-IgG). 1G10 showed reduced binding to FVIII in the presence of all tested A2-specific mAbs. A reduction in binding of phage 1G10 to FVIII could result from a competition of phage and A2-specific mAb for the same epitope. Among the tested A2-specific mAbs, GMA012 and 1D4 almost completely blocked binding of phage clone 1G10 to FVIII. Recently a set of A2-specific mAbs including GMA012 and 1D4 was analysed for their epitope specificity by homolog-scanning mutagenesis (16). Based on these results and ELISA competition experiments a Venn diagram was designed, categorizing the mAbs into different groups with individual epitopes. GMA012 bound to amino acid residues 497–510 and 584–593 on both native and denatured FVIII and belongs to group D of A2-specific mAbs. As a member of group E 1D4 has its epitope in the region of amino acids 604–740. Both groups comprise antibody binding to the C-terminal region of the A2-domain. Based on the observed competition pattern scFv 1G10 might be categorised as a group DE antibody. However, since phage particles have a length of approximately 930 nm (11), reduced binding of phage 1G10 in presence of an A2-specific mAb could also be due to steric hindrance. Besides, binding of an individual mAb to the FVIII A2 domain can also lead to conformational changes of distant binding sites. Therefore, reduction in binding of 1G10 not necessarily proves that 1G10 and the respective mAb have overlapping binding sites. To eventually categorise 1G10 as member of the DE group the epitope of this scFv has to be mapped more precisely, for example by homolog-scanning mutagenesis as described by Markovitz et al. (16).

To further evaluate the nature of the 1G10 epitope, binding of 1G10 to FlrFVIII before and after 30 min incubation at 56°C was compared (Fig. 2b). While B domain-specific phage 1C6 bound equally to native and denatured FVIII, binding of phage 1G10 was strongly decreased after heat denaturation. Hence, binding of 1G10 to FVIII is conformationally dependent.

**scFv-hlgG constructs**

**Cloning and expression**

To convert the scFv 1G10 into the format of the different human IgG subclasses, the sequence of 1G10 was cloned into hlgG1-hlgG4 expression vectors, each coding for the hinge as well as the C32 and C33 region of the hlgG heavy chain. 1G10-hlgG fusion proteins were transiently expressed in HEK-293T cells and purified via protein A or G chromatography. The purity of the preparations was confirmed by coomassie staining, as exemplarily shown for the purification of 1G10-hlgG1 (Fig. 3). The monomer of 1G10-hlgG1 had a molecular weight of about 54 kDa and a slightly higher apparent molecular weight on the gel. The antibody was not detectable in the flow through and the wash fraction of the protein A column. Eluates 1 to 6 showed a decreasing protein amount with the 1G10-hlgG1 monomer being the prominent polypeptide. In the 1G10-hlgG1 concentrate two minor polypeptide chains appear below 36 kDa and 28 kDa, probably derived from degradation products of the antibody. Purification and characterisation of 1G10-hlgG2–4 yielded comparable results (data not shown).

Concentration-dependent binding of 1G10-hlgG1 to FVIII was verified by ELISA (Fig. 4a). As phage 1G10 competed with the inhibitory murine mAb 1D4 (7000 BU/mg) for binding to FVIII (see again Fig. 2a), the potential of 1G10-hlgG1 to inhibit FVIII activity was analysed by Bethesda measurements (Fig. 4b). The results of this analysis showed that 1G10-hlgG1 inhibits the function of FVIII. A specific inhibitory potency of approximately 50 BU/mg was calculated. FVIII inhibitors are classified in type I and type II inhibitors, according to their neutralisation kinetics. In contrast to inhibitors of type I, type II inhibitors do not completely inhibit FVIII activity at high (saturating) concentrations (1). The obtained data set for 1G10-hlgG1 does not allow a categorisation in type I or type II inhibitors. For that, measurement of the residual FVIII activity at higher and eventually saturating concentrations of antibody would be necessary.

**Conclusion**

FVIII-specific scFvs were successfully isolated from synthetic phage display libraries. The antibody fragments showed different binding properties to FVIII and derived fragments. For three scFvs FVIII domain specificity could be determined: scFv 186 and presumably also 1C6 bind to the B domain, and 1G10 binds to the A2 domain of FVIII.

More precisely, 1G10 probably binds to the C-terminal region of the A2 domain and belongs to group DE of A2-specific mAbs, the potential of 1G10 to FVIII is conformationally dependent.
AS the scFv competes with GMA012 (group D) and 1D4 (group E) for binding to FVIII, scFv 1G10 was successfully converted into the format of the four human IgG subclasses as shown for 1G10-hlgG1. The respective 1G10-hlgGs were expressed in mammalian HEK-293T cells and purified by protein A or G chromatography. 1G10-hlgG proteins still binds to FVIII and therefore can be used as standard to quantitatively determine FVIII-specific IgG subclasses in patients’ plasma. Further monitoring of the FVIII-specific IgG subclass distribution of haemophilia A patients undergoing ITI will help to elucidate a potential relation between FVIII-specific IgG subclasses and the outcome of ITI. Furthermore, collected data can be analysed relating to a possible correlation of treatment-related factors and FVIII-specific subclass development. In combination the results of future analyses could have an impact on the treatment of haemophilia A patients undergoing ITI and make tolerance induction more successful.

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Conflict of interest

The authors declare, that there is no relevant conflict of interest.

References

4. Fulcher CA, de Graaf Mahoney S, Roberts JR et al. Localization of human factor FVIII inhibitor epi-

What is known about this topic?

- Haemophilia A patients with FVIII inhibitors show different FVIII-specific IgG subclass distributions.
- FVIII-specific IgG subclass levels are measured differently and first results indicate that IgG subclass contributions correlate to outcome of ITI.

What does this paper add?

- Selection of FVIII-specific single chain variable fragments (scFvs) from synthetic phage display libraries.
- Characterisation of selected scFvs and cloning into the context of human IgG subclasses for the use as FVIII-specific IgG subclass standards.

Fig. 3 SDS-PAGE analysis of 1G10-hlgG1 purification: 1G10-hlgG1 was purified by protein A chromatography. Input (0.01‰ of total amount), flow through (FT), wash fraction, eluates, and 0.5 µg concentrate (Conc.) were analysed by SDS-PAGE and coomassie stained. MW, molecular weight.

Fig. 4 1G10-hlgG1 binds to FVIII and inhibits FVIII activity

a) Binding of 1G10-hlgG1 to FVIII was analysed in ELISA. Error bars represent the SD of the mean absorption of triplicates.
b) Residual FVIII activity in per cent of control in the presence of 1G10-hlgG1 was measured in the Bethesda assay.