In-depth comparison of N-glycosylation of human plasma-derived factor VIII and different recombinant products: from structure to clinical implications

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To cite this article: Canis K, Anzengruber J, Garenaux E, Feichtinger M, Benamara K, Scheiflinger F, Savoy L-A, Reipert BM, Malisauskas M. In-depth comparison of N-glycosylation of human plasma-derived factor VIII and different recombinant products: from structure to clinical implications. J Thromb Haemost 2018; 16:1592–603.

Essentials

- Glycosylation heterogeneity of recombinant proteins affects pharmacokinetics and immunogenicity.
- N-glycomics/glycoproteomics of plasma-derived Factor VIII and 6 recombinant FVIII s were compared.
- Depending on cell line, significant differences to plasma-derived FVIII were observed.
- Recombinant FVIII s expressed distinct and immunologically relevant epitopes.

Summary. Background/Objective: Human factor VIII (FVIII) is a plasma glycoprotein, defects of which result in hemophilia A. Current substitution therapy uses FVIII products purified from human plasma or from various cell lines (recombinant FVIII) with different levels of B-domain deletion. Glycosylation is a post-translational protein modification in FVIII that has a substantial influence on its physical, functional and antigenic properties. Variation in glycosylation is likely to be the reason that FVIII products differ in their pharmacokinetics, pharmacodynamics and immunogenicity. However, the literature on FVIII glycosylation is inconsistent, preventing assembly into a coherent model. Seeking to better understand the glycosylation mechanisms underlying FVIII biology, we studied the N-glycosylation of human plasma-derived (pd)FVIII and six rFVIII products expressed in CHO, BHK or HEK cell lines. Methods: FVIII samples were subjected to head-to-head detailed glycomic and glycoproteomic characterization using a combination of MALDI-MS and MS/MS, GC-MS and UPLC-UV-MSE technologies. Results/Conclusion: The results of our study detail the N-glycan repertoire of pdFVIII to an unprecedented level, and for the first time, provide evidence of N-glycolyneuraminic acid (NeuGc) found on pdFVIII. Although site-specific glycosylation of rFVIII proved consistent with pdFVIII regardless of the expression system, the entire N-glycan content of each sample appeared significantly different. Although the proportion of biologically important epitopes common to all samples (i.e. sialylation and high-mannose) varied between samples, some recombinant products expressed distinct and immunologically relevant epitopes, such as LacdiNAc (LDN), fucosylated LacdiNAc (FucLDN), NeuGc, LewisX/Y and Galα1,3Gal epitopes. rFVIII expressed in HEK cells showed the greatest glycomic differences to human pdFVIII.

Keywords: blood proteins; factor VIII; hemophilia A; N-glycosylation; recombinant proteins.

Introduction

Human factor VIII (FVIII) is a plasma glycoprotein critical for the function of the blood coagulation cascade, serving as a cofactor for FIXa in the conversion of FX to FXa [1,2]. FVIII is a heterodimer composed of one heavy (HC) and one light (LC) chain joined by a non-covalent linkage, which requires a divalent metal ion. Both chains display a domain structure ordered as A1-a1-A2-a2-B (HC) and a3-A3-C1-C2 (LC). Along the biosynthetic and secretion pathway, FVIII is subjected to complex post-translational protein modifications, including N- and O-linked glycosylation as well as tyrosine sulfation [3]. A deficiency of active FVIII results in
hemophilia A, one of the most common severe bleeding disorders [4].

N-glycosylation is highly complex, diverse and influential post-translational protein modification, and is involved in countless critical biological processes at a cellular and molecular level [5]. N-glycosylation is initiated in the endoplasmic reticulum by an oligosaccharide precursor attaching to any asparagine residue within the consensus sequon Asn-Xxx-Ser/Thr (being any amino acid except proline). Each precursor is subjected to specific and complex maturation along the secretory pathway, via regulated activity of numerous glycosidases and glycosyltransferases conferring definite properties to the sugar moieties. Glycosylation has a substantial influence on the physical and functional properties of proteins, and thus is a critical quality attribute for the efficacy and safety of biopharmaceuticals [6,7].

Hironaka et al. (1992) first reported N-glycans associated with human plasma-derived FVIII (pdFVIII) [8]. The authors chemically released N-glycans from the protein backbone, separated them, and structurally characterized them using a combination of paper electrophoresis, lectin affinity, size exclusion chromatography, sequential glycosidase treatment and methylation analysis. The N-glycan profile determined was dominated by sialylated complex-type bi-antennary core-fucosylated oligosaccharides, along with lower levels of high-mannose, tri-antennary and tetrantennary species. Some of the complex-type structures characterized contained blood group epitopes and traces of LewisX determinants. Because oligosaccharides were released prior to analysis, this study provided no information on the distribution of these N-glycans along the pdFVIII amino acid sequence.

Twenty years later and using a combination of capillary liquid chromatography and mass spectrometry techniques, Kannicht et al. reported the N-glycan content of four separate glycosylation sites located within the A and C domains of pdFVIII. Glycosylation sequons N60, N258, N1829 and N2137 were shown to be substituted by a distinctive population of structures, which included complex-type, high-mannose or hybrid structures [9]. However, the B-domain enclosing 19 of 25 potential FVIII N-glycosylation sequons was not analyzed [3].

Recombinant protein technology has allowed recombinant FVIII (rFVIII) products for treatment of hemophilia to be generated from mammalian cell lines, including Chinese hamster ovary (CHO), baby hamster kidney (BHK) and human embryonic kidney (HEK) cells. Cell lines used to produce recombinant glycoproteins have different glycosylation capacities because of different sets of expressed glycosyltransferases that fluctuate not only according to species but also according to tissue or cell type [10–12]. Furthermore, the protein structure itself and cell culture factors such as media components and culture conditions can affect the glycosylation machinery [12].

Numerous publications suggest that variations in glycosylation affect the pharmacokinetics, pharmacodynamics and immunogenicity of biopharmaceuticals. Therefore, robust structural characterization and determination of an optimal glycosylation pattern are important during development and manufacturing of recombinant glycoproteins. We sought to build a comprehensive and understandable model of the N-glycosylation of FVIII and understand its implications. Unfortunately, because of the distinct approaches, methods and technologies previously used to determine FVIII glycosylation, it is virtually impossible to compare published datasets generated at different points in time.

Our study aimed for highly detailed description and head-to-head comparison of the N-glycosylation of human pdFVIII and six commercial rFVIII products expressed in various cell lines. We first assessed the N-glycan population associated with each sample using MALDI-MS technologies, and examined the nature and proportion of high-mannose or complex-type structures to determine cell-specific glycosylation abilities. In-depth MALDI-MS/MS fragmentation analyses revealed significant differences between samples via characterization of specific terminal epitopes such as ABH blood groups, Lewis antigens, N-glycolyneuraminic acid (NeuGc) and Galα1,3Gal. Finally, seeking to provide insight into the influence of N-glycans on FVIII physical and biochemical properties, we performed UPLC-UV-MS analyses to outline the glycan content associated with each glycosylation site along the sequence of pdFVIII or rFVIII.

Material and methods

Samples

Samples included plasma-derived FVIII (pdFVIII) purified from a commercially available lyophilized pdFVIII product and six commercial rFVIII products as described in Table 1. All samples were prepared and analyzed in parallel according to identical procedures. The reproducibility and robustness of each method were demonstrated by processing sample replicates. Sample B was prepared and analyzed in duplicate in two independent sets of experiments, sample D in duplicate within one experiment.

Purification of pdFVIII

Von Willebrand factor (VWF)-free pdFVIII was purified from a commercially available lyophilized pdFVIII product. Multiple vials were reconstituted and pooled to achieve a homogeneous starting material. FVIII was captured on an anti-FVIII affinity column and further processed using strong cation exchange chromatography. Finally, another purification step on a strong anion exchange resin was performed for buffer exchange and protein concentration.

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Proteolytic digestion

Each sample was dialyzed overnight against 50 mM NH₄HCO₃/1 mM CaCl₂ before sequentially undergoing reduction using dithiothreitol, alkylation using iodoacetamide and digestion using trypsin (Promega, Madison, WI, USA). One aliquot of the tryptic digest was directly subjected to UPLC-UV-MSE analysis (see last section).
and another was further processed for glycomic analyses as follows.

**Enzymatic N-glycan release and purification**

The tryptic digest was first purified using a Sep-Pak C18 cartridge (Waters, Milford, USA). The cartridge was conditioned with methanol (MeOH), propanol (PrOH) and then 5% aq CH₃COOH before sample loading and clean-up using 5% aq CH₃COOH. Tryptic digest was eluted using 20% aq and 60% aq PrOH, dried-down, then resuspended in 50 mM NH₄HCO₃ and digested using PNGase F (Roche, Basel, Switzerland). The released N-glycans were purified on a Sep-Pak SPE C18 cartridge using the procedure described above, but collecting and drying-down the post-loading 5% aq CH₃COOH fraction.

**N-glycan permethylation and sample clean-up**

Dry N-glycans were resuspended in a slurry of NaOH, dimethyl sulfoxide (DMSO) and methyl iodide (ICH₃), and left to incubate under shaking. The reaction was stopped using H₂O and permethylated N-glycans were extracted using CHCl₃, then purified on a reverse-phase C18 Sep-Pak cartridge. The cartridge was conditioned using MeOH, acetonitrile (ACN) and H₂O before sample loading. Permethylated N-glycans were washed using H₂O and 15% aq ACN, then eluted using 35% aq and 50% aq ACN and finally dried-down.

**MALDI-TOF MS and MS/MS N-glycan profiling**

MALDI-MS and MS/MS analyses were performed using a 5800 MALDI-TOF/TOF mass spectrometer (Sciex, Framingham, MA, USA). Permethylated N-glycans were resuspended in MeOH and then spotted with 2,5-dihydroxybenzoic acid matrix onto a target plate. MS analyses were performed in the reflectron positive ion mode within the m/z range 1200–6000. At least 3000 acquisitions were accumulated to produce each MS spectrum. For MS/MS analyses, timed ion selector was on and collision energy was set to 1 kV. At least 4000 acquisitions were accumulated to generate each MS/MS spectrum.

**GC-MS inter-monosaccharide linkage analysis**

The composition of each sample in terms of monosaccharides and the nature of the inter-monosaccharide linkages were characterized by GC/MS analysis of partially methylated and acetylated alditols acetates (PMAA) prepared from permethylated N-glycans as follows. Following hydrolysis in 2M aq trifluoroacetic acid (TFA), deuteroreduction using sodium borodeuteride (NaBD₄) and acetylation using acetic anhydride, the resulting PMAA were extracted using CHCl₃, dried-down and resuspended in hexane. Analyses were performed using a Clarus 680 GC/600T MS (PerkinElmer, Waltham, MA, USA) fitted with a RTx-5MS column.

**UPLC-UV-MS² N-glycan site distribution**

Analyses were performed using an Acquity UPLC I-Class in line with a Xevo G2-S Q-TOF mass spectrometer (Waters Corporation, Milford, MA, USA). Tryptic digests were separated on an Acquity BEH C18 column using 0.1% aq TFA as solvent A and 0.1% TFA in ACN: H₂O (90 : 10) as solvent B. MS acquisition was performed in Electrospray-MS² positive ion mode over the range m/z 100–3000.

**Fig. 2.** Outline of the N-glycan populations characterized from human plasma-derived and recombinant FVIII samples. BHK, CHO, HEK.

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Glycomics of plasma-derived FVIII

The pdFVIII N-glycans were characterized using MALDI-MS and MS/MS technology in combination with GC-MS data. The MALDI-MS profile generated shows 28 distinct [M+Na]+ signals consistent with each high-mannose, hybrid and complex-type N-glycan structure (Fig. 1A and B). The high-mannose and hybrid groups, regarded as poorly processed N-glycans, constitute a small proportion of the population, whereas the complex-type N-glycans that undergo significant tissue-specific maturation are the main and most diverse class of structures (Fig. 2). The profile is dominated by fucosylated and sialylated bi-antennary N-glycans (m/z 2605, 2779 and 2967) with lower amounts of tri- and tetra-antennary species. The pdFVIII complex-type N-glycan antennae were exclusively of type 2 N-acetyllactosamine (LacNAc; Galα1-4GlcNAc) and to be widely subjected to further maturation to specific peripheral epitopes. It was first estimated that two-thirds of the complex-type N-glycan antennae are modified by addition of a terminal sialic acid residue (Fig. 2), of which one-third is linked through a α2,3 pattern and two-thirds through an α2,6 pattern (Fig. 3). Although the vast majority of sialic acid residues were shown to be N-acetylglycolニュリミン酸 (NeuGc; Fig. 1B; m/z 2635 and 2997). MALDI MS/MS fragmentation data further demonstrated the presence of NeuGc via detection of diagnostic fragment ions (Fig. 4A; fragments m/z 406, 877 and 2142). Based on MS data, NeuGc was estimated to represent ~1% of the

| Antennae type | Plasma-derived FVIII | Sample A | Sample B | Sample C | Sample D | Sample E | Sample F |
|---------------|----------------------|----------|----------|----------|----------|----------|----------|
| Type of LacNAc unit | Type 2 | Type 2 | Type 2 | Type 2 | Type 2 | Type 2 | Type 2 |
| PolyLacNAc elongations | + | + | – | – | – | + | – |
| LacdiNAc antennae | – | – | – | – | – | + | + |
| α2,3 sialic acid (%) | 34 | 95 | 100 | 100 | 77 | 48 |
| α2,6 sialic acid (%) | 66 | 5 | 0 | 0 | 6 | 23 | 52 |
| N-glycoylneuraminic acid (%) | 1.3 | 2.8 | 1.6 | 2.9 | 4.8 | – | – |
| ABO blood groups | + | – | – | – | – | – | – |
| Lewisα epitope | – | + | – | + | – | + |
| Lewisβ epitope | – | – | – | – | – | + |
| FucLDN epitope | – | – | – | – | – | + |
| Galα1-3Gal antigen | – | + | – | – | – | – |
| Bisecting GlcNAc | – | – | – | – | – | + |

Fig. 3. Biologically significant structural N-glycan features identified in human plasma-derived and recombinant FVIII samples. Blue and red shading indicate significant similarity and differences to pdFVIII, respectively. [Color figure can be viewed at wileyonlinelibrary.com]
total pdFVIII sialic acid content. ABO(H) blood group epitopes (GalNAc\textsubscript{2,3}(Fuc\textsubscript{2,6})Gal\textsubscript{b}, Gal\textsubscript{2,3,6}(Fuc\textsubscript{2,6})Gal\textsubscript{b} and Fuc\textsubscript{2,6}Gal\textsubscript{b}) were the second major class of peripheral epitopes identified (see Figs 3 and 4B).

No evidence for other human terminal epitopes was detected. The main structural features of pdFVIII complex-type N-glycans therefore include predominant fucosylation of the core and significant antennae capping with either sialic acids (66%) or ABO(H) blood group antigens (10%). Based on these results, a schematic representation of human FVIII N-glycan maturing along the secretory pathway is proposed in Fig. 5.

Glycoproteomics of plasma-derived FVIII

N-glycan distribution along the pdFVIII amino acid chain was analyzed by UPLC-UV-MS\textsuperscript{E}. The amino acid sequence revealed 25 N-glycosylation consensus sequons (N-X-S/T where X is any amino acid except P).

Glycosylation was observed for 18 sequons, and the N-glycan content of each sequon examined (Fig. 5, bottom panel). For clarity and comparability with recombinant samples, sequons were grouped and referred to as Site 1 (N\textsubscript{60}), Site 2 (N\textsubscript{2137}), B-domain (all sequons from residue 760 to 1667), Site 3 (N\textsubscript{1829}) and Site 4 (N\textsubscript{2137}).

The N-glycan content of Site 1 had the most abundant fucosylated and sialylated bi-antennary structures, which dominated the whole N-glycan profile (Fig. 1B; see \textit{m/z} 2605, 2779 and 2967). Comparable glycan contents were determined for each of the 14 occupied sequons of the B-domain with relatively low variability. The content on Site 3 was similar in nature but had discreet variations in proportion as a result of a significantly higher level of ABO(H) blood group epitopes than any other sequon. In contrast to sites 1 and 3, the N-glycan content of Site 2 was restricted to hybrid and short non-fucosylated complex-type structures, which suggests a low propensity of the surrounding domain to glycan maturation. Similarly,
Site 4 showed a unique content exclusively restricted to non-processed high-mannose structures.

Glycomic comparability of plasma-derived FVIII with recombinant FVIII material

The six commercial rFVIII products studied comprised two full-length, two B-domain-deleted, one B-domain-truncated and one chimeric construct, produced in various cell lines, including CHO, BHK and HEK (Table 1).

For each sample, a population of 20–30 N-glycans was identified and characterized as shown in Figures S1–S6. As reported for pdFVIII, high-mannose and complex-type N-glycans predominated, with only few or no hybrid structures. The relative content of high-mannose differed significantly from sample to sample, reflecting the presence or absence of the B-domain (Fig. 2). In all samples, the complex-type species were dominated by core-fucosylated bi-antennary structures accompanied by minor tri- and tetra-antennary forms. However, sample A provided stronger signals reflecting large structures, whereas samples E and F showed no or minor analogous signals, indicating that CHO, BHK and HEK cells display significant variations in the activity of their respective pool of GlcNac transferases. These results suggest that CHO cells produce N-glycans with an average number of antennae closest to human pdFVIII, BHK cells produce a higher number and HEK a lower number (Fig. 2).

Identification of capping structures demonstrated the highest dissimilarity between pdFVIII and rFVIII N-glycosylation. Although peripheral epitopes were mainly restricted to sialylation and ABO(H) blood groups on pdFVIII, various distinct structures were characterized on recombinant samples, reflecting additional distinct cell-specific maturation processes.

First, when sialic acids were the most prevalent capping residues, their nature and linkage differed significantly between samples. When NeuAc sialylation was the most common feature on samples produced in BHK and CHO cells, the linkage pattern differed considerably from pdFVIII, with an almost exclusive α2,3 linkage pattern, reflecting the inability of these cell lines to generate α2,6 sialylation [13]. In contrast, samples produced in HEK cells exhibited both α2,3 and α2,6 linkage, and a much lower level of sialylation (Fig. 3). In addition, a proportion of NeuGc residues was detected within the total sialic acid content in all samples expressed in BHK and CHO cells, but not in those produced in HEK cells (Fig. 3).

Most other peripheral structures characterized from recombinant samples included fucosylated epitopes. The LewisX epitope (Gal\(_{b1,4}(\text{Fuc}_{a1,3})\text{GlcNAc}\)) was detected at low levels in samples A and C and in abundance in samples E and F. Also, low levels of LewisA epitope (\(\text{Fuc}_{a1,2}\)-Gal\(_{b1,4}(\text{Fuc}_{a1,3})\)GlcNAc) were recorded in sample E.

In addition, in samples E and F, which express LacdiNAc antennae, the α1,3-fucosyl transferase activity involved in the formation of LewisX led to fucosylated LacdiNAc epitopes (\(\text{FucLDN; GalNAc}_{b1,4}(\text{Fuc}_{a1,3})\)GlcNAc), as illustrated in Fig. 6B.

Data generated from sample D also revealed another specific peripheral epitope defined as Gal\(_{a1,3}\)Gal\(_{b}\) (Figure S4; m/z 2809 and 3620). MS/MS fragmentation data confirmed the nature of this antigen, as shown in Fig. 6A. Based on MS data, Gal\(_{a}\)Gal was estimated to comprise ~2% of the antennae in sample D, and was not detected in the other samples.

Overall, the data demonstrated a degree of similarity between pdFVIII and rFVIII produced in BHK and CHO cells, with one, not minor, distinction in the expression of fucosylated peripheral epitopes. In contrast, rFVIII produced from HEK cells showed highly distinctive N-glycan patterns with significant differences, such as the level of antennae capping, proportion of antennae sialylation and expression of epitopes normally absent from the human bloodstream.

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Glycoproteomic comparability of plasma-derived and recombinant FVIII material

Sequence correspondence between pdFVIII and rFVIII samples confirmed the previous designations, bearing in mind that samples C to F are B-domain deleted. For samples A and B, N-glycosylation was observed for 19 of 25 putative N-glycosylation sequons (i.e. the 18 sequons occupied on pdFVIII plus N847). For samples C to F, N-glycosylation was detected in four of six putative N-glycosylation sequons (Fig. 7; sites 1–4).

For each rFVIII sample, the N-glycosylation profile of Site 1 and each of the B-domain occupied sequons (samples A and B only) was consistent with the full profile as determined by MALDI-MS (Figures S1–S6 and Fig. 7). In contrast, Site 3 showed a divergent glycosylation pattern with overexpression of distinctive peripheral epitopes such as Lewis epitopes for samples A, E and F, GalαGal for sample D, and LDN/FucLDN for samples E and F. As discussed, this observation may reflect good accessibility of the domain surrounding Site 3 to Golgi enzymes related to glycan peripheral capping. As reported for pdFVIII, Site 2 of each rFVIII sample was associated with high-mannose or poorly-processed complex-type structures, and Site 4 was exclusively occupied by high-mannose structures for all rFVIII samples.

Taken together, these glycoproteomic data suggest that site-specific glycosylation is remarkably conserved across pdFVIII and rFVIII products, irrespective of protein model and cell line used for manufacturing (Fig. 7). This suggests a strong conservation of the early glycosylation enzymatic machinery, which notably contrasts with the divergence of late glycan processing enzymatic activities highlighted in Fig. 3.

Discussion

Human plasma-derived FVIII N-glycosylation

Having characterized nearly 30 different N-glycan compositions, we have detailed the N-glycan repertoire of

![Fig. 7. Site-specific N-glycan distribution of plasma-derived and recombinant FVIII samples. Semi-quantitative results were calculated based on extracted ion chromatograms generated from UPLC-UVMS data. The three major N-glycans found at each site examined are indicated, together with their respective abundance expressed in % occupancy of each site. Monosaccharide symbols are as described in Fig. 1A. [Color figure can be viewed at wileyonlinelibrary.com]](https://example.com)
pdFVIII to an unprecedented level. The MALDI-MS profile generated was consistent with most human plasma glycoprotein profiles, with dominating sialylated and core fucosylated bi-antennary complex-type structures together with secondary populations of high mannose, tri-antennary and tetra-antennary complex-type N-glycans [14] (Fig. 1A and B).

Regardless of high mannose species comprising 16% of the population, sialic acid appeared to be the most prevalent pdFVIII N-glycans terminal epitope, detected in 67% of complex-type antennae in both α2,3 and α2,6 linkages (34% and 66%, respectively; Figs. 2 and 3). Terminal sialic acids are commonly associated with an increase in the in vivo half-life of plasma glycoproteins, as they prevent exposure of underlying galactose or N-acetylgalactosamine residues. These residues are recognized with high affinity by the asialoglycoprotein receptor in the liver and are rapidly cleared from circulation [15]. It should be noted, however, that further FVIII clearance mechanisms, including interaction through sialic acid-recognizing receptors such as Siglec-5, have been reported. Furthermore, clearance can be modulated through high mannose and galactosylated glycans binding to C-type lectin member 4 family M (CLEC4M) and galectins Gal-1 and Gal-2 [16]. When NeuAc was the most abundant sialic acid detected in antennae, ~1% proportion of N-glycolylneuraminic acid was also documented for the first time on pdFVIII. Terminal NeuGc is widely expressed in most mammals except humans because these species have an irreversible mutation in the CMAH gene encoding the cytidine monophosphate-N-acetylated neuraminic acid hydroxylase responsible for the biosynthesis of NeuGc [17]. Consequently, NeuGc may be highly antigenic, as reflected by the abundance of anti-NeuGc immunoglobulins in human plasma [18]. Conversely, an increasing number of publications report traces of NeuGc on endogeneous glycoproteins, including VWF, and it is now accepted that NeuGc can be metabolically incorporated from dietary sources and transferred onto human glycoproteins [19,20].

ABO(H) blood groups were the second class of pdFVIII N-glycan terminal epitope identified. FVIII is one of the few circulating glycoproteins associated with ABO(H) epitopes, together with VWF [21]. VWF and FVIII share a similar glycomic profile, including features such as predominant core fucosylation and sialylation, similar proportions of ABO(H) antigens (10–15%) and low levels of polyLacNAc elongations and bisected GlcNAc [22]. Several studies have indicated a direct influence of the ABO(H) blood type on plasma VWF levels, and also on FVIII activity [23,24]. The influence of ABO(H) epitopes on FVIII N-glycans on the clearance of FVIII, however, is not known.

Glycoproteomic microheterogeneity analyses revealed that 18 of 25 putative pdFVIII N-glycosylation sequons (Asn-Xxx-Ser/Thr) were occupied. These results indicate that the N-glycan content was relatively homogeneous and representative of the global profile shown in Fig. 1(B), except for three particular sequons. The content on Site 3 (N1829) revealed a significantly higher level of terminal ABO(H) blood group epitopes than for the other sites. Based on published FVIII crystal structures, the oligosaccharide group at N1829 is exposed on the surface of the molecule, suggesting good accessibility of the surrounding domain to late-Golgi enzymatic activities responsible for peripheral capping of the glycans [25,26]. Similarly, protein–protein or glycan–protein interactions could also have an advantage around Site 3 in the A3 domain of FVIII [27]. The content of Site 2 was restricted to hybrid and short non-fucosylated complex-type structures, which implies, in contrast to Site 3, the surrounding domain’s low propensity to glycan maturation. Similarly, Site 4 revealed a unique content exclusively restricted to non-processed high-mannose structures. In fact, data on FVIII crystal structures previously demonstrated that the sugar moieties of sites 2 and 4 are poorly exposed and located in a domain interface within the molecule [25,26].

Recombinant FVIII N-glycosylation

N-glycosylation of rFVIII samples produced in CHO, BHK or HEK cells has been described as differing from that of pdFVIII, mainly in terminating epitopes, such as sialic acids, ABH blood group and Lewis epitopes, Galα1,3Gal antigens and LacdiNAc residues.

As with pdFVIII, sialylation was the most common terminal structure on rFVIII samples manufactured from BHK and CHO cells, although the linkage pattern differed, with an almost exclusive α2,3 sialic acid linkage, reflecting this cell line’s inability to generate α2,6 sialylation [13]. In contrast, the level of sialylation of samples produced from HEK cells was much lower but exhibited both α2,3 and α2,6 linkage. As discussed above, terminal sialic acids are related to an increased glycoprotein half-life in plasma by preventing exposure of residues recognized by the asialoglycoprotein receptor responsible for clearance [15]. Also, proportions of NeuGc residues comparable to pdFVIII were detected within the total sialic acid content of samples expressed in rodent cells (1.6–4.8% on rFVIII vs. 1.3% on pdFVIII), but not in samples produced in human cells (Fig. 3).

ABH blood group epitopes were not identified on any rFVIII N-glycan, regardless of expression cell system, in this study or previously published studies [8,9,28,29]. In contrast, LewisX epitopes were detected at low levels in samples derived from BHK and CHO cells (samples A and C) and in abundance in samples produced from HEK cells. Low levels of LewisX epitope were also recorded in sample E produced from HEK cells. Lewis epitopes are naturally present in humans but not commonly found on circulating glycoproteins. Instead, these epitopes are mostly expressed in the central nervous system and on mucins, mediating several critical functions.
including cell signaling, apoptosis, adhesion and migration [30,31]. The biological consequence of such epitopes on plasma glycoproteins remains unknown.

The non-human mammalian glycan epitope Galα1,3Gal (alpha-Gal) was detected on N-glycans from sample D, expressed in CHO cells, and was absent in all other samples analyzed in this study. Humans have circulating antibodies against alpha-Gal depending on pre-exposure to xenotypic proteins [32,33]. A severe anaphylactic reaction to these non-human glycan epitopes derived from a therapeutic Sp2/0-based antibody (cetuximab) was reported in a patient who appeared to have high pre-existing anti-alpha-Gal antibodies [34]. In a comparative pharmacokinetics study of pdFVIII and BHK cell-derived rFVIII in baboons no differences in half-life in blood circulation were observed, suggesting that antibodies against alpha-Gal, if present, do not interact with rFVIII in plasma, possibly because of the interference of VWF [8].

Moreover, terminal epitopes LDN and FucLDN were previously detected on the antenna of HEK cell-produced rFVIII [9] and confirmed by our results, as well as on HEK cell-produced rFVII [10]. These epitopes were not detected on pdFVIII and are not usual in human circulating glycoproteins, except for pituitary hormones, in which they are critical in regulating clearance [35]. LDN has been instead reported on glycodelin and in samples derived from various types of tumors [36,37]. Furthermore, LDN and FucLDN epitopes are not commonly detected in vertebrate, but in pathogen sources, and are therefore usually associated with immune recognition [38–40]. At the same time, terminal GalNAc residues as found in LDN-type glycans are known to be a potent ligand for asialoglycoprotein receptor mediation [15].

In conclusion, the results of our study suggest that the site-specific N-glycosylation pattern of all six tested rFVIII samples was consistent with the pdFVIII model despite major differences in overall glycosylation and terminating epitopes as described above (Fig. 7). Moreover, the data confirm that different cell lines have distinct glycosylation capacities depending not only on species but also on tissue type from which the cells are derived [10–12]. We therefore recommend assessing the suitability of a cell line in expressing human therapeutic glycoproteins on an individual basis, as long-term administration of rFVIII containing galcan epitopes that are not common on human plasma proteins might possibly, although no incident has been reported yet, affect a drug’s efficacy and safety.

Addendum

K. Canis and M. Malisauskas designed the research; K. Canis, E. Garenaux and M. Feichtinger performed the experiments; K. Canis, J. Anzengruber, E. Garenaux and M. Malisauskas interpreted the results; K. Canis, J. Anzengruber and E. Garenaux prepared the manuscript; F. Scheiflinger, B.M. Reipert, L.-A. Savoy and K. Benamara revised the intellectual content. All authors approved the final manuscript.

Acknowledgements

This work was financially supported by Shire. The authors thank J. Pichot for his substantial contribution to the experimental and analytical effort.

Disclosure of Conflict of Interests

SHIRE sponsored SGS M-Scan SA for this study. K. Canis, E. Garenaux and L.-A. Savoy are employees of SGS M-Scan SA. J. Anzengruber, M. Feichtinger, B.M. Reipert and F. Scheiflinger are employees of Shire. K. Benamara and M. Malisauskas were employees of Shire at the time of this study.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. MALDI-TOF mass spectrum of permethylated N-glycans isolated from Sample A. Structural assignments and monosaccharide symbols are as described in Fig. 1.

Fig. S2. MALDI-TOF mass spectrum of permethylated N-glycans isolated from Sample B. Structural assignments and monosaccharide symbols are as described in Fig. 1.

Fig. S3. MALDI-TOF mass spectrum of permethylated N-glycans isolated from Sample C. Structural assignments and monosaccharide symbols are as described in Fig. 1.

Fig. S4. MALDI-TOF mass spectrum of permethylated N-glycans isolated from Sample D. Structural assignments and monosaccharide symbols are as described in Fig. 1.

Fig. S5. MALDI-TOF mass spectrum of permethylated N-glycans isolated from Sample E. Structural assignments and monosaccharide symbols are as described in Fig. 1.

Fig. S6. MALDI-TOF mass spectrum of permethylated N-glycans isolated from Sample F. Structural assignments and monosaccharide symbols are as described in Fig. 1.

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