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Characterisation of the post-translational modifications of a novel, human cell line-derived recombinant human factor VIII

Christoph Kannicht [a,*], Margareta Ramström [b], Guido Kohla [a], Maya Tiemeyer [c], Elisabeth Casademunt [c], Olaf Walter [d], Helena Sandberg [b]

[a] Octapharma, Berlin, Molecular Biochemistry, Germany
[b] Octapharma, Stockholm, Sweden
[c] Octapharma, Heidelberg, Germany
[d] Octapharma, Lachen, Switzerland

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ABSTRACT

Introduction: Host cell lines used for recombinant protein expression differ in their ability to perform post-translational modifications (PTMs). The currently available recombinant human FVIII (rhFVIII) products are produced in mammalian, non-human cell lines. For rhFVIII, glycosylation and sulfation are vital for functionality and von Willebrand factor (VWF)-binding affinity. Here we present the characterisation of the PTMs of a novel, human cell line-derived recombinant human FVIII (human-cl rhFVIII). rhFVIII expression in a human cell line avoids expression of undesirable mammalian glycoforms like Galα1-3Galβ1-GlcNAc-R (α-Gal) and *N*-glycolylneuraminic acid (Neu5Gc), which constitute epitopes antigenic to humans.
Materials and methods: We describe sulfation analysis, glycan profiling and characterisation using liquid chromatography-mass spectrometry and high performance anion exchange chromatography with pulsed amperometric detection.
Results and conclusions: Human-cl rhFVIII is confirmed to be sulfated and glycosylated comparable to human plasma-derived FVIII. Most importantly, human-cl rhFVIII is devoid of the antigenic Neu5Gc or α-Gal epitopes observed in Chinese Hamster Ovary- and Baby Hamster Kidney-derived rFVIII products. Both the avoidance of non-human glycan structures and the achievement of complete sulfation are proposed to lower the intrinsic immunogenicity of human-cl rhFVIII compared with current rFVIII products.

Introduction

Haemophilia A (HA) results from functional deficiency of endogenous Factor VIII (FVIII), a key plasma protein in the blood coagulation cascade [1]. Untreated, this produces recurrent haemorrhage into joints and muscles, causing debilitating arthropathies and long-term morbidity [2]. Exogenous FVIII treatment restores haemostatic control, limiting further damage – in severe disease (FVIII activity<1%) FVIII prophylaxis has been the mainstay of treatment for decades [1,3,4].

Commercial FVIII products have advanced during the last 50 years and viral safety has greatly improved [1,4,5]. Today, multiple FVIII products are available, within two broad classes: human plasma-derived (pd) FVIII, pooled and concentrated from human donor blood; and recombinant human FVIII (rhFVIII). The manufacturing approach addressed here combines recombinant protein production with an entirely human expression system, promising a 'third generation' of FVIII replacement products [6].

Of the currently available rhFVIII products, two are full-length FVIII proteins and one is B-domain deleted; all are produced in mammalian, non-human cell lines [7]. Experience with cross species-produced recombinant human proteins suggests that non-human cellular processing can affect protein functionality, impacting upon efficacy and raising a product's immunogenic potential (for review see [8,9]). Prominent examples of the impact of cross-species recombinant protein production include altered pharmacokinetics, i.e. lower recovery and decreased half-life of Chinese Hamster Ovary (CHO)-cell derived recombinant FIX compared to pdFIX [10], and the α-Gal-driven immune reaction against a monoclonal antibody produced in the mouse myeloma cell line SP2/0 [11].

Despite vast improvements overall in HA care, concentrate shortages, inadequate access to affordable treatment in developing countries and

Abbreviations: α-Gal, Galα1-3Galβ1-GlcNAc-R; Asn/N, asparagine; BHK, baby hamster kidney; CHO, Chinese hamster ovary; EIC, extracted ion chromatogram; FVIII, factor VIII; HA, Haemophilia A; HEK, human embryonic kidney; HexNAcHexNAc, hexosamine-hexosamine; HPAEC-PAD, High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection; human-cl rhFVIII, human cell line recombinant human factor VIII; ITI, immune tolerance induction; KDN, 2-keto-3-deoxy-d-glycero-d-galactonononic acid; LC, liquid chromatography; LDNF, fucosylated LacdiNAc; MS, mass spectrometry; MS/MS, tandem MS; Neu5Gc, *N*-glycolylneuraminic acid; Neu5Ac, *N*-acetylneuraminic acid; pdFVIII, plasma-derived FVIII; PTM, post-translational modifications; r(h)FVIII, recombinant (human) FVIII; TOF, time of flight; Tyr/Y, tyrosine; VWF, von Willebrand factor.

* Corresponding author at: Octapharma R&D, Molecular Biochemistry Berlin, Walther-Nernst-Str. 3, D-12489 Berlin, Germany. Tel.: +49 30 8322 7360; fax: +49 30 8410 9862.
E-mail address: christoph.kannicht@octapharma.com (C. Kannicht).

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greater product immunogenicity, mean that many HA patients receive sub-optimal FVIII therapy, both in medical and economic terms [5].

A recent survey of HA patients and carers evaluated key concerns regarding FVIII products [12,13]. With twenty-five years of pathogen-safe FVIII products largely eliminating former anxieties over viral transmission, the current major issue in this HA population is product immunogenicity, i.e. the likelihood of developing a FVIII inhibitor [13]. This was echoed by physicians in a similar survey of American Haematologists published in 2008, which ranked '*reduced possibility that inhibitor titre may rise*' directly behind '*time required to stop bleeding*' and '*quick pain relief*' [14]. FVIII inhibitors are a difficult clinical complication in HA [15]. Although decades of research and several immune tolerance induction (ITI) studies have improved inhibitor management, it remains a serious complication for the patient and an expensive one for healthcare providers [16–18].

Various host cell lines are available for recombinant protein expression – e.g. bacterial, yeast, fungal, plant, insect and mammalian. Individual cell lines differ in yield, cost, specificity and stability, but most importantly, in their ability to perform post-translational modifications (PTMs). For rhFVIII products, glycosylation and sulfation are most vital [19]. De-glycosylation of rhFVIII with endoglycosidases results in substantial loss of clotting activity, reduced to $62 \pm 13\%$ compared with $94 \pm 14\%$ for native rhFVIII, and sulfation of key residues has been shown to be vital for FVIII functionality and von Willebrand factor (VWF)-binding affinity [19,20]. Formerly, glycosylation was simply considered an inconvenient obstacle to protein purification and analysis; however, the key contribution of glycobiology to protein structure is now increasingly recognised, with glycoengineering a growing field of study [8,9,21]. Glycosylation is species- and organ-specific [8,9,22]. Inter-species glycosylation capacity diverges with evolutionary distance from humans. Hence the more primitive cell lines which are frequently used in recombinant protein technology, tend to incorporate non-human, antigenic glycan moieties [22]. Organ-specific glycosylation differences, whilst not immunologically pertinent, may engender functional differences, and the impact of other important intra-species epitopes, e.g. ABO-blood group determinants, must also be considered [23].

Numerous examples exist of biotherapeutic proteins whose *in vivo* efficacy and stability appear mediated by their native glycosylation profile [21]. Co-translational biosynthetic processing of glycan chains is associated with quality control of nascent protein molecules, such that improper glycosylation may disrupt cellular trafficking, flagging proteins for degradation rather than secretion. Incorrect glycoforms may also produce misfolding due to absent oligosaccharide markers, leading to less stable, non-functional conformations [8,9,22].

Several genetic and non-genetic risk factors for inhibitor development in response to FVIII therapy have been identified (for review see [24,25]). Amongst the concentrate-related risk factors, differences in the primary structure (e.g. non-haemophilic, non-synonymous single nucleotide polymorphisms (SNPs) [26]) or in protein length (e.g. full-length vs. B-domain-deleted FVIII) have been reported to impact on immunogenicity [27,28] and are currently the subject of ongoing studies [29]. Depending on the patient's HLA class II repertoire, even the type of B-domain deletion as a potential carrier of specific T-cell epitopes can affect the immunogenic risk [24].

Increased inhibitor incidence in recent decades has been linked to the introduction of rFVIII products and amongst other factors, a role for the association of FVIII with VWF in circulation has been suggested [23,30]. In the context of PTMs, two particular characteristics of FVIII, the VWF-binding affinity and the presence of antigenic glyco-epitopes, may contribute to the product-related development of a FVIII immune response.

The stability of infused FVIII is dependent on its native VWF binding properties, which in turn are dependent on FVIII PTMs, particularly the sulfation of specific residues [20]. Clinical evidence to date remains equivocal; however an international, open-label, uncontrolled, non-interventional, multi-centre observational study investigating the immunogenicity of recombinant and human plasma concentrates is in progress [1,15,31,32]. Mouse studies have demonstrated higher levels of FVIII inhibitors following administration of hamster cell-derived rhFVIII products compared to pdFVIII [33]. Furthermore, addition of VWF to the hamster cell-derived rhFVIII product reduced anti-FVIII induction [33] and it has been suggested that VWF decreases the immunogenicity of circulating FVIII by reducing the uptake of unbound FVIII by antigen presenting cells, preventing initiation of an immune response [33]. Following infusion, FVIII products with high VWF-affinity are able to efficiently bind endogenous VWF, forming the stable FVIII-VWF complex. Interestingly, Lin *et al.* (2004) evaluated different FVIII concentrates for their VWF binding capacity and found that, unlike the plasma-derived products, the evaluated rFVIII concentrates contained a fraction of FVIII:Ag molecules unable to associate with VWF [34]. Interim data from an on-going good clinical practice (GCP) study in previously untreated patients showed a remarkably low inhibitor rate (5.1%) in patients treated solely with pdFVIII containing VWF [35].

With no non-human cell line able to authentically replicate human PTMs, cell lines for biopharmaceutical protein expression must be carefully matched to the demands of the application [36,37]. Prokaryotic organisms, e.g. *Escherichia coli (E. coli)* usually do not produce *N*-linked glycans, and simple eukaryotes, e.g. yeasts, are limited to high-mannose oligosaccharide synthesis [8,22]. Accordingly, the initial cloning of FVIII 25-years ago was performed in mammalian, non-human cell lines, heralding the first generation of rhFVIII products derived from Chinese Hamster Ovary (CHO) cells, or Baby Hamster Kidney (BHK) cells. Since then, a slow process of consolidation and expansion in availability of recombinant products derived from mammalian, non-human cell lines has improved product purification and performance. Production of rhFVIII in mammalian cell lines offered scalable production, good clotting efficacy and reduced viral-safety concerns [38]. However, accurate PTM profiles in recombinant therapeutic glycoproteins have increasingly been documented to optimise functionality, activity and half-life, whilst reducing antigenicity and immunogenicity; particularly with regard to an appropriate oligosaccharide profile, excluding potentially antigenic epitopes [39–41]. Consequently, the next step in improving the efficacy and safety of recombinant therapeutic human protein is to establish expression platforms derived from the target organism, i.e. humans, thereby avoiding principal inter-species PTM differences [42–44].

Human-cell Line Recombinant Human FVIII (human-cl rhFVIII)

A rhFVIII has been produced in a human cell line with human-specific post-translational protein processing [6]. This aims to combine the native physiological advantages of human pdFVIII concentrates with the scalable supply and production of recombinant FVIII products. Human-cl rhFVIII is produced in the absence of animal derivatives and undergoes five chromatographic purification steps, including two virus inactivation/removal steps, maximising viral and pathogenic safety [6,42]. Analysis of the novel B-domain deleted human-cl rhFVIII product indicates a fully sulfated glycoprotein with an oligosaccharide and sialic acid composition comparable to human pdFVIII. Results of glycan-profiling and sulfation analysis using chromatographic and mass spectrometric (MS) techniques are presented and discussed in a physiological context.

Materials and Methods

FVIII Samples

Several batches of human-cl rhFVIII were produced in-house. Human pdFVIII was purified from Octanate® (Octapharma AG, Lachen, Switzerland) using monoclonal antibody affinity chromatography, and