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Regular Article

Physicochemical characterisation of rVIII-SingleChain, a novel recombinant single-chain factor VIII

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ABSTRACT

rVIII-SingleChain is a novel recombinant single-chain factor VIII (FVIII) construct, comprising covalently bonded heavy and light chains. Post-translational modifications of FVIII affect physicochemical parameters, including hydrophobicity and charge. The most relevant post-translational modifications of FVIII products are *N*-glycosylation of asparagine residues and tyrosine sulphations. Here, the physicochemical properties, thrombin cleavage products and post-translational modifications of rVIII-SingleChain were investigated and compared against commercially available recombinant FVIII (rFVIII) products with a predominant two-chain structure (B-domain deleted rFVIII and full-length rFVIII). rVIII-SingleChain was expressed in Chinese hamster ovary (CHO) cells and purified by chromatographic methods. Physicochemical properties of rVIII-SingleChain or thrombin-derived cleavage products were assessed using size-exclusion chromatography, reversed-phase chromatography and sodium dodecyl sulphate polyacrylamide gel electrophoresis. Analysis of the respective carbohydrate structures was performed after release of *N*-glycans by PNGase F followed by fluorescence labelling and high-performance liquid chromatography. Proteolysis by trypsin generated the corresponding peptides, which were analysed for sulphated tyrosines by liquid chromatography-electrospray ionisation time of flight-mass spectrometry. rVIII-SingleChain was shown to be of high purity and homogeneity, and presented a well-defined single-chain molecule with predominant β-sheet conformation. The coagulation-relevant thrombin-activation products of rVIII-SingleChain were comparable with those obtained by activation of commercially available rFVIII products. rVIII-SingleChain post-translational modifications were similar to other CHO cell-derived rFVIII products for *N*-glycopattern and tyrosine sulphation. In conclusion, rVIII-SingleChain is of high homogeneity and purity, and provides an expected cleavage pattern on activation, setting the basis for optimal efficacy in the patient.

Summary of work

What is known about recombinant single-chain factor VIII	What this paper adds to the scientific literature
• The mainstay of clinical management for patients with haemophilia A is replacement of the deficient factor VIII (FVIII) with plasma-derived or recombinant factors.	• rVIII-SingleChain expressed in Chinese hamster ovary (CHO) cells can be isolated as a homogeneous, highly pure compound.
• Current commercially available recombinant FVIII preparations have a two-chain design.	• Confirmation that coagulation-relevant thrombin-activation products of rVIII-SingleChain are similar to those of commercially available two-chain FVIII products.

(*continued*)

Summary of work

What is known about recombinant single-chain factor VIII	What this paper adds to the scientific literature
• rVIII-SingleChain is a novel recombinant single-chain FVIII construct.	• Evidence that rVIII-SingleChain post-translational modifications are similar to other CHO-derived recombinant FVIII products for *N*-glycopattern and tyrosine sulphation.

Introduction

Haemophilia A, the most common form of haemophilia, is an X-linked recessive congenital bleeding disorder caused by deficient or defective coagulation factor VIII (FVIII). Patients with this condition

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have bleeding episodes in joints and tissues and, if untreated, experience long-term morbidity [1].

FVIII is a two-chain glycoprotein that circulates in plasma in a complex with von Willebrand factor (VWF). The molecular weight of full-length FVIII is approximately 280 kDa and it is composed of a heavy chain (domains A1a1 A2a2B) and a light chain (domains a3A3C1C2), which are held together by non-covalent interactions [2]. Trace amounts of thrombin, generated during the initiation of coagulation, activate FVIII and lead to specific cleavages to form a trimeric complex consisting of A1a1, A2a2 and the *N*-terminally shortened light chain (A3C1C2).

The management of patients with bleeding disorders has improved dramatically in recent decades, resulting in enhanced life expectancy and quality of life [3]. For haemophilia A, the mainstay of clinical management is currently replacement of the deficient FVIII with plasma-derived or recombinant factors, either on demand or as prophylaxis [4].

Since recombinant FVIII (rFVIII) was first used in the 1980s, continuing efforts to improve the safety of replacement therapy have led to the latest generation of recombinant products being devoid of animal- or human-derived proteins in the cell culture or formulation processes [5]. Despite these advances, which have provided a normal or near-normal life expectancy for most people with haemophilia, key challenges remain [6]. Approximately 30% of patients undergoing FVIII replacement therapy develop inhibitory antibodies, rendering treatment ineffective and increasing the risk of bleeding complications [7–9]. Thus, there is a strong medical need for rFVIII therapy that offers reduced potential for immunogenicity. In addition, research has focused on developing rFVIII products with an extended half-life to improve treatment convenience and standard of care for people with haemophilia A [10].

rVIII-SingleChain is a novel recombinant single-chain FVIII construct. In contrast to other commercially available rFVIII preparations, which are two-chain molecules (i.e. B-domain deleted [BDD] rFVIII and full-length rFVIII), rVIII-SingleChain has a single-chain design in which a truncated B-domain covalently links the heavy and light chains. As the three predominant thrombin cleavage sites are not modified in rVIII-SingleChain, it is expected that following activation by thrombin, the rFVIIIa produced from rVIII-SingleChain will be structurally comparable to that formed from two-chain full-length rFVIII or BDD rFVIII (Fig. 1).

rVIII-SingleChain was designed as a single-chain FVIII molecule with improved intrinsic stability. In addition, it has been shown that the affinity of rVIII-SingleChain for VWF is markedly higher, and its pharmacokinetic properties are improved, compared with full-length rFVIII [11].

In this study, we characterised rVIII-SingleChain with regard to purity, structure and post-translational modifications. Thrombin cleavage products were also investigated to confirm formation of proper activation products. Results were compared against commercially available rFVIII products.

Materials and Methods

Materials

rVIII-SingleChain was expressed in Chinese hamster ovary (CHO) cells. Harvests were processed by several chromatographic and virus reducing steps to obtain the purified active ingredient, which was further formulated and freeze-dried to obtain the drug product. Commercially available BDD and full-length rFVIII products were used as comparators (ReFacto® AF, Pfizer Limited, Kent, UK; Advate®, Baxter AG, Vienna, Austria). For analysis, the rFVIII products were reconstituted according to the manufacturers' instructions.

Size-exclusion High-performance Liquid Chromatography (SE-HPLC)

Size distribution of FVIII samples was investigated by SE-HPLC using a Beckman liquid chromatography instrument (Beckman Coulter GmbH System Gold, Detector Shimadzu RF-10A XL). The Cosmosil 5 Diol-300-II column used (WICOM Germany GmbH, 64646 Heppenheim, Germany; 7.5 × 600 mm; molecular weight range: 10 000–700 000 Da) allowed detection of high molecular weight components (HMWCs) in rFVIII preparations. In each run, 40 IU of FVIII (activity according to the chromogenic substrate (CS) FVIII activity assay, Chromogenix Coamatic FVIII

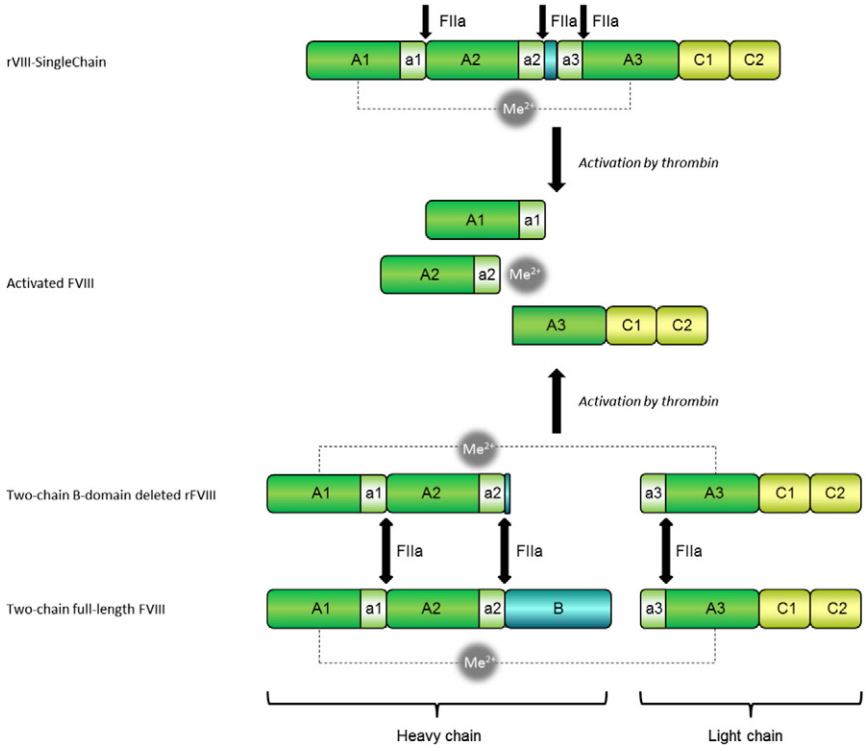

Fig. 1. Schematic activation cleavages of rVIII-SingleChain and two-chain rFVIII. rFVIII = recombinant factor VIII; FIIa = factor IIa (thrombin).