Cellular Immunology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Research paper

Biochemical characterization and immunogenicity of Neureight, a recombinant full-length factor VIII produced by fed-batch process in disposable bioreactors

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ARTICLE INFO

Keywords:
Hemophilia A
Factor VIII
Immunogenicity
Disposable fed-batch production technology
CHO cell line

ABSTRACT

Hemophilia A is a X-linked recessive bleeding disorder consecutive to the lack of circulating pro-coagulant factor VIII (FVIII). The most efficient strategy to treat or prevent bleeding in patients with hemophilia A relies on replacement therapy using exogenous FVIII. Commercially available recombinant FVIII are produced using an expensive perfusion technology in stainless steel fermenters. A fed-batch fermentation technology was recently developed to produce 'Neureight', a full-length recombinant human FVIII, in Chinese hamster ovary (CHO) cells. Here, we investigated the structural and functional integrity and lack of increased immunogenicity of Neureight, as compared to two commercially available full-length FVIII products, Helixate and Advate, produced in baby hamster kidney or CHO cells, respectively. Our results demonstrate the purity, stability and functional integrity of Neureight with a standard specific activity of 4235 ± 556 IU/mg. The glycosylation and sulfation profiles of Neureight were similar to that of Advate, with the absence of the antigenic carbohydrate epitopes α-Gal and Neu5Gc, and with sulfation of Y1680, that is critical for FVIII binding to von Willebrand factor (VWF). The endocytosis of Neureight by human immature dendritic cells was inhibited by VWF, and its half-life in FVIII-deficient mice was similar to that of Advate, confirming unaltered binding to VWF. *In vitro* and *in vivo* assays indicated a similar immunogenicity for Neureight, Advate and Helixate. In conclusion, the production of full-length FVIII in a fed-batch fermentation mode generates a product that presents similar biochemical, functional and immunogenic properties as products developed using the classical perfusion technology.

1. Introduction

Hemophilia A is a X-linked recessive bleeding disorder consecutive to the lack of circulating pro-coagulant factor VIII (FVIII) [1]. Treatment and prevention of bleedings in patients with hemophilia A rely on replacement therapy using either plasma-derived or recombinant FVIII [2]. FVIII is among the most expensive protein therapeutics on the market owing to the low yields of production of the glycoprotein in the case of recombinant products and to the different steps required to eliminate potential viral agents [3]. The poor stability of FVIII [4,5] is an additional constraint that participates in increase of production costs. Production processes for recombinant FVIII (both full-length or B domain-deleted) are all based on expensive perfusion technology in stainless steel fermenters [6–8]. The use of continuous perfusion technology allows the growth of FVIII-producing cells to high concentrations while ensuring a relatively short protein residence time [9,10]. Shorter residence times were proposed to allow efficient production and less disruption of the recombinant FVIII (rFVIII) protein [11], thus minimizing the generation of potentially immunogenic FVIII degradation products. To address the need for better supply of affordable rFVIII at global level, efforts have focused on cell line development, process improvements and optimization of expression rates in commercial mammalian cell culture processes. Nevertheless, expression does typically not exceed 0.1 mg FVIII/L cell culture supernatant for a full-length FVIII [12].

We recently developed a fed-batch fermentation technology based on disposable fermentation technology to produce a full-length recombinant human FVIII. Compared to perfusion technology, fed-batch

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https://doi.org/10.1016/j.cellimm.2018.05.002
Received 14 February 2018; Received in revised form 4 May 2018; Accepted 4 May 2018

processes have significant advantages, such as ease of process validation and characterization, better lot definition and consistency, ease of downstream processing, overall reduced time to product approval, reduced medium consumption, reduced waste generation, less manpower requirements [9,13], easier technology transferability and less automation requirements. Current state-of-the-art single-use disposable technologies have been recognized as an increasing trend in biopharmaceutical manufacturing due to several advantages like reduction of the risk for cross-contamination, elimination of cleaning steps and an increase in flexibility, ease for handling and an estimated reduction in costs between 20 and 40% [14–16]. In the present work, the new full-length FVIII, referred to as Neureight, was compared to available full-length recombinant products, and the immunogenicity was assessed in *in vitro* and *in vivo* assays.

2. Material and methods

2.1. Sources of FVIII and VWF

Neureight is a recombinant full-length FVIII produced in eukaryotic CHO cells using a fed-batch process in a disposable setting in the absence of any stabilizing agents such as of VWF or human serum albumin. The purification of Neureight involves a step of affinity chromatography using the VIIIselect matrix (GE Healthcare, Piscataway, NI, USA) followed by traditional ion exchange, a hydrophobic interaction chromatography steps and virus reduction steps such as detergent treatment and nanofiltration. Helixate and Advate were purchased from CSL-Behring (Marburg, Germany) and Shire (Vienna, Austria), respectively. For *in vitro* studies, the three full-length FVIII were reconstituted in their respective excipients and dialyzed against DMEM-F12 for 2 h at 4 °C. FVIII were then aliquoted and stored at −80 °C until use. FVIII:Ag and FVIII:C in aliquots were assessed by Asserachrom (Stago, Asnières sur Seine, France) and by chromogenic assay (Dade-Behring, Marburg, Germany), respectively. Total protein content was measured by Bradford using bovine serum albumin as a standard. FVIII levels are indicated as International Units (IU) per ml based on the FVIII:Ag levels. Wilfactin (LFB, Les Ulis, France) was used a source of VWF. It was dialyzed in DMEM-F12 during 2 h at 4 °C, and aliquoted and stored at −80 °C.

2.2. Electrophoretic and Western blot analyses

SDS-PAGE was performed using 4–12% Bis-TrisNuPage gels (Life Technologies, Carlsbad, California, USA) followed by a staining with a Silver Express Stain Kit (Life Technologies). Western blotting was carried out using an anti-FVIII antibody GMA-012 (Green Mountain Antibodies, Burlington, VT, USA) which recognizes the A2 domain epitopes at residues 497–510 and 584–593, followed by an alkaline phosphatase conjugated anti-mouse antibody (Sigma-Aldrich Chemie GmbH, Munich, Germany).

2.3. Glycan profiling

MALDI-TOF MS analysis of N-glycans was performed after deglycosylation and derivatization of the separated and purified N-glycans. The permethylated glycans were further purified by a C18 cartridge and lyophilized, solubilized with 50% v/v methanol:water and mixed with MALDI mass spectrometry matrix before analysis. Positive ion reflectron MALDI mass spectra were acquired on a VOYAGER DE PRO (AB Sciex). Relative intensities (%) of N-glycans were determined based on the peak heights of the corresponding deisotoped monoisotopic mass ions. Interpretation of glycan structures corresponding to monoisotopic masses was performed using ExPASyGlycoMod tool and GlycoWork Bench.

2.4. Identification and assignment of sulfation sites, glycosylation sites and glycopeptides

Tryptic digestion and detection of the sulfo- and glycopeptides by MS and tandem MS (MS/MS) was performed to elucidate the sulfation and glycosylation sites and the corresponding glycans. The yielded tryptic glycopeptides were purified and enriched for the respective analysis by MALDI-TOF MS where positive ion linear MALDI mass spectra were acquired in different mass ranges and with multiple acquisition conditions on a MALDI-TOF/TOF Autoflex III spectrometer (Bruker Daltonics). Further, the separated glycopeptide-enriched fractions were deglycosylated, and the yielded peptides were also analyzed by MALDI-TOF MS where positive ion reflectron and linear MALDI mass spectra were acquired in different mass ranges and with multiple acquisition conditions on a MALDI-TOF/TOF Autoflex III spectrometer (Bruker Daltonics). This was done for determining which of the theoretical N-glycosylation sites are actually occupied with N-glycans.

For identification of sulfation sites, the obtained deglycosylated peptides were also injected on a nano-HPLC system coupled to an ion trap nano-electrospray mass spectrometer (LTQvelos, ThermoScientific). Acquisition of mass data was performed in a mass range of 300 to 2000 Da excluding monocharged ions. Proteome Discoverer ThermoElectron (v: 1.4) was used to analyze acquired LC-MS/MS data files.

2.5. Preparation of monocyte-derived dendritic cells

Blood from healthy donors was anonymously obtained from Etablissement Français du Sang Ile-de-France (Ivry-sur-Seine, France). Monocytes from peripheral blood mononuclear cells were isolated using anti-CD14 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) from heparinized buffy coats of healthy donors. Purified monocytes were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, L-glutamine and antibiotics in the presence of 500 IU/10^6 cells of IL-4 (R&D Systems, Lille, France) and 1000 IU/10^6 cells GM-CSF (ImmunoTools, Friesoythe, Germany). After 5 days, the non-adherent immature dendritic cells (DCs)-enriched fraction was harvested and the immature status was confirmed by the expression of surface phenotypic markers (data not shown).

2.6. Incubation of FVIII with MO-DCs

For maturation experiments, immature 5-day old MO-DCs ($2.5x10^5$ cells/well) were incubated in 500 µl of X-VIVO^{15} (Lonza) with the different FVIII preparations at 1 or 10 IU/ml for 48 h. Surface expression of CD80, CD83, CD86, CD40 and HLA-DR was investigated by flow cytometry on 10,000 acquired cells per condition. As a positive control for induction of maturation, we used LPS at 100 ng/ml. Cells incubated alone were used as a negative control.

For FVIII endocytosis experiments, immature MO-DCs (4×10^5 cells/well) were incubated for 120 min with the FVIII at 50 to 400 IU/ml in 200 µl of X-VIVO^{15} at 4 °C or 37 °C. Intracellular FVIII was recognized after permeabilization with 0.5% saponin, using the FITC-labeled monoclonal antibody (anti-A2 FVIII domain mAb77IP52H7, 10 µg/ml, a kind gift from LFB, Les Ulis, France). 10,000 cells were acquired for each condition for flow cytometry analysis. Percentages of cells positive for FVIII were then calculated. Uptake was quantified as the difference in the % cells at 37 °C and 4 °C. When indicated, FVIII (400 IU/ml, 215 nM) was pre-incubated in X-VIVO^{15} with VWF (87.5 IU/ml, 3.6 µM) for 20 min at room temperature, prior to incubation with MO-DCs. mAb77IP52H7 does not interfere with the interaction of FVIII with VWF (not shown).

2.7. FVIII-specific T-cell activation assay

Five-day old immature MO-DCs derived from monocyte of a healthy