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Functional characteristics of the novel, human-derived recombinant FVIII protein product, human-cl rhFVIII

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ABSTRACT

Introduction: Hemophilia A is routinely treated by administration of exogenous coagulation factor VIII (FVIII). As safety and efficacy of FVIII products have improved over the years, development of FVIII-neutralizing antibodies (FVIII inhibitors) has emerged as the most serious complication. The new human cell line-derived recombinant human FVIII (human-cl rhFVIII) is the first recombinant FVIII product produced in a human cell line without additive animal proteins, with a goal of minimizing the risk of inhibitor development.
Materials and methods: Biochemical analyzes of purity, molecular and functional attributes of the novel human-cl rhFVIII were undertaken for product characterization.
Results and conclusions: Human-cl rhFVIII was shown to be highly pure, with host-cell protein and DNA traces comparable to, or lower than, currently marketed recombinant FVIII (rFVIII) products. Human-cl rhFVIII was shown to have high specific FVIII activity and characteristics similar to full-length rFVIII products. Furthermore, no significant discrepancy between one-stage and chromogenic assay results were observed for human-cl rhFVIII, indicating potency ratios of these assays comparable to the full-length rFVIII products. In functional tests, human-cl rhFVIII exhibited physiological thrombin generation and a normal rate of inactivation by activated protein C. Importantly, human-cl rhFVIII displayed higher binding capacity with von Willebrand factor than comparator products, thus minimizing circulating unbound FVIII and further reducing the potential risk of inhibitor development.

Introduction

Factor VIII (FVIII) is a crucial cofactor in the intrinsic coagulation pathway, where factor X (FX) is converted to activated FX (FXa) by activated factor IX (FIXa). Human FVIII is a ~300 kDa glycoprotein of 2332 amino acids with the domain structure A1-A2-B-A3-C1-C2 [1–4]. A and C domains account for hemostatic function, however the B-domain is dispensable in this respect [5–7]. Following gene translation, FVIII protein undergoes extensive modifications, including *N*-glycosylation, *O*-glycosylation and tyrosine sulfation [4]. Prior to secretion, FVIII is cleaved into a metal-ion bridged heterodimer, consisting of a heavy chain (~200 kDa), comprising the A1-A2-B domains, and a light chain (~80 kDa) comprising the A3-C1-C2 domains [6,7]. Secreted FVIII is rapidly bound non-covalently to von Willebrand factor (VWF), a multifunctional, multimeric, and heterogeneous plasma protein of 0.6-20 MDa with disulfide-linked subunits of approximately 255 kDa [8]. VWF stabilizes FVIII by preventing proteolytic degradation and considerably prolongs *in vivo* survival time [5,9,10].

FVIII requires activation by thrombin to serve as a cofactor in the coagulation process. Trace quantities of thrombin are sufficient for activation, through limited proteolysis of the FVIII protein generating a labile complex of three subunits; a 50 kDa and a 43 kDa polypeptide, corresponding to the A1 and A2 domains, respectively; and a 73 kDa protein fragment, generated by cleavage of the *N*-terminal of the light chain that removes the VWF-binding site, resulting in VWF dissociation [2,11–13]. VWF dissociation exposes the phospholipid binding site located within the FVIII C2 domain, allowing

Abbreviations: APTT, activated partial thromboplastin time; ANOVA, analysis of variance; APC, activated Protein C; BDD, B-domain deleted; BHK, baby hamster kidney; BSA, bovine serum albumin; CHO, Chinese hamster ovary; ETP, endogenous thrombin potential; FVIII(a), (activated) factor VIII; FVIII:Ag, factor VIII antigen; FVIII:C, factor VIII activity; FIX(a), (activated) factor IX; FX(a), (activated) factor X; HA, Hemophilia A; HCP, host cell proteins; HCT, High capacity trap; HRP, horse-radish peroxidase; Human-cl rhFVIII, human cell line recombinant human factor VIII; HEK, human embryonic kidney; IS, International standard; (HP)LC, (high performance) liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; pdFVIII, plasma-derived FVIII; PTM, post-translational modifications; r(h)FVIII, recombinant (human) FVIII; TOF, time of flight; SEC, size-exclusion chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide electophoresis; RSD, relative standard deviation; SPR, surface plasmon resonance; Y, tyrosine; VWF, von Willebrand factor.

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formation of the tenase complex (activated FVIII [FVIIIa], FX and FIXa) on negatively-charged phospholipid surfaces made available by activated platelets and leads to FX activation [14,15]. *In vivo* regulation of FVIIIa is accomplished by its natural inhibitor, activated protein C (APC), which effects inactivation via proteolytic cleavage in the presence of its cofactor, protein S [12,16].

Decreased physiological levels of FVIII results in hemophilia A (HA), a genetic disorder associated with prolonged bleeding [17]. Restoration of coagulation capacity through replacement with exogenous FVIII is an effective HA therapy [17]. Currently, the mainstay of therapy is prophylaxis with FVIII products, either plasma-derived (pdFVIII) or recombinant (rFVIII). Full-length and B-domain-deleted (BDD) rFVIII products are available; both provide excellent efficacy and pathogen safety in the treatment of HA [17]. Pathogen transmission was historically a concern, however improvements in screening and production methodology mean that pathogen-safe products have now been available for over 25 years [17]. Consequently, development of FVIII inhibitors, a serious complication in HA treatment, is currently a main area of concern [18,19]. FVIII inhibitors are circulating allo-antibodies that block one or more epitopes important for FVIII function, rendering infused FVIII ineffective in restoring hemostasis [17–20]. Following treatment with current rFVIII products, derived from Chinese hamster ovary (CHO) or baby hamster kidney (BHK) cells, the cumulative risk of inhibitors in severe HA patients is up to 39% [21].

All currently available rFVIII products are produced in hamster cells and contain trace amounts of non-human proteins [22]. FVIII synthesized in these cells undergoes hamster-specific post-translational modifications (PTMs) and since FVIII undergoes extensive, specific PTMs, this is theoretically sub-optimal. Human-cl rhFVIII (Octapharma AG, Switzerland) now in clinical trials, is the first rFVIII product to be produced in a human-cell line (human embryonic kidney 293F [HEK293F]). Contrary to the more commonly used HEK293T cell line variant, the HEK293F cell line used for the production of human-cl rhFVIII does not contain or express the oncogenic SV40 Large T-antigen. Since the cell cultivation is performed using a defined serum-free medium and no animal- or human-derived materials are added during production, the risk for virus contamination in the final product would be negligible. The human-cl rhFVIII purification process includes five chromatographic steps and two virus clearance steps with solvent/detergent treatment and nanofiltration to destroy/remove any theoretically occurring enveloped or non-enveloped viruses. The prime advantage of using a human cell line for rhFVIII expression is the human-like pattern of PTMs obtained, akin to that found on endogenous FVIII molecules. This study reports on the purity, molecular, and *in vitro* functional characteristics of human-cl rhFVIII, with particular emphasis on features considered to improve function and reduce immunogenicity.

Materials and Methods

Sample batches

Up to 26 different batches of human-cl rhFVIII were submitted to various analyzes. As comparators, three batches of the pdFVIII product Octanate® (Octapharma AG, Switzerland), ten batches of Kogenate® and its successor product Kogenate FS® (Bayer, USA), two batches of Advate® (Baxter, USA) and five batches of the BDD rFVIII product ReFacto® and its successor products ReFacto AF®/Xyntha® (Wyeth, USA), were analyzed.

Residual DNA analysis

Residual host-cell DNA was analyzed using DNA-threshold method for total DNA. The Threshold® DNA assay kit, (Molecular Devices, USA) was used to determine residual host-cell DNA in the test sample. The use of the kit ensures the detection of all single-stranded DNA molecules ≥100 base pairs, i.e. fragments below the minimal size of a functional gene, which is estimated to be approximately 200 base pairs [23].

Host cell protein (HCP) analysis

For the analysis of residual HCPs, polyclonal anti-HCP antibodies were raised in rabbits. The HCPs for immunization were cultivated in HEK 293F mock cells, transfected with the identical vector as used for transfection of FVIII-expressing cells, but lacking the FVIII-expression cassette. Microtitre plates were coated with polyclonal anti-HCP antibodies. After incubation with the samples, horseradish peroxidase (HRP)-conjugated anti-HCP antibodies were added to bind to captured HCP, creating a 'sandwich' complex. HCP content of the test samples was calculated from a standard curve.

Measurement of protein concentration

Protein concentration was determined by measuring fluorescence emission at 340 nm upon excitation at 280 nm. The assay was used to determine concentrations in the range of 10–30 μg/mL where fluorescence emission of human-cl rhFVIII was linear. Measurements were performed using a standard of human-cl rhFVIII with a well-defined protein concentration determined by amino acid composition analysis.

FVIII activity measurement

Factor VIII activity (FVIII:C) was determined by chromogenic assay based on the Coatest SP® FVIII kit (Chromogenix/Instrumentation Laboratory, USA) in 96-well microplates on a Freedom EVO® robotic sample processor (Tecan, Switzerland) according to the requirements of the European Pharmacopoeia. In addition, a one-stage clotting assay was performed on a BCS® XP instrument using Dade FVIII Deficient Plasma and Actin®, Activated Cephaloplastin Reagent (Siemens Healthcare Diagnostics Products, Germany). All samples were assayed against the current WHO international standard (IS) for FVIII concentrates (7th or 8th IS). Three vials of each sample/batch were assayed on three separate occasions using five replicates/vial. Standard and samples were pre-diluted to 1 IU/mL in congenital FVIII-deficient substrate plasma (Helena Laboratories, USA) before final dilutions in Tris-NaCl buffer + 1% BSA. The assays were evaluated as parallel-line bioassays, relating log concentration (x) to log response (y) using validated software.

Determination of total protein of human-cl rhFVIII for calculation of specific activity was based on fluorescence spectroscopy as described above.

FVIII antigen measurement

Factor VIII antigen (FVIII:Ag) was determined using an enzyme immunoassay (ELISA) for FVIII, Asserachrom® FVIII:Ag kit (Diagnostica Stago, France) with substitution of Tris-NaCl buffer + 1% bovine serum albumin (BSA) for sample dilutions in place of the provided kit buffer and using the 5th IS for FVIII/VWF in plasma, or a product-specific standard calibrated against the 5th IS instead of the kit standard in the case of human-cl rhFVIII. Standard and samples were pre-diluted to 1 IU/mL in Tris-NaCl buffer + 1% BSA before final dilutions to 1/20, 1/40 and 1/80 in the same buffer. Samples were assayed at least in duplicate, in each of two separate assays. Evaluation was performed using the parallel line concept as above.

Thrombin generation

The Thrombogram-thrombinoscope assay (Thrombinoscope BV, The Netherlands) used was based on fluorometric detection of a peptide substrate, which undergoes thrombin-mediated conversion into a fluorescent compound, thus enabling monitoring of the kinetics of thrombin generation.