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Optimisation of the Factor VIII yield in mammalian cell cultures by reducing the membrane bound fraction

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ABSTRACT

In vivo, clotting Factor VIII (FVIII) circulates in plasma bound to von Willebrand factor (vWF), and the vWF:FVIII complex prevents binding of FVIII to phosphatidylserine (PS). Activation of FVIII by thrombin releases FVIII from vWF, and subsequently FVIII binds to PS exposed on activated platelets and forms the tenase complex together with clotting Factor IX. *In vitro*, during serum free production of recombinant FVIII (rFVIII), production cells also expose PS, and since vWF is not present to hinder interaction of secreted rFVIII with PS, rFVIII is partly associated with the cell membrane of the production cells. Recently, we showed that as much as 90% of secreted rFVIII is bound to transiently transfected production cells during serum free conditions. In this study, we investigated the effect of including vWF in the serum free medium, and demonstrate that addition of vWF results in release of active membrane bound rFVIII to the culture medium. Moreover, the attachment of rFVIII to cell membranes of un-transfected HEK293 cells was studied in the presence of compounds that competes for interactions between rFVIII and PS. Competitive assays between iodinated rFVIII (^{125}I-rFVIII) and annexin V or ortho-phospho-L-serine (OPLS) demonstrated that annexin V and OPLS were able to reduce the membrane bound fraction of rFVIII by 70% and 30%, respectively. Finally, adding OPLS to CHO cells stably expressing FVIII increased the yield by 50%. Using this new knowledge, the recovery of rFVIII could be increased considerably during serum free production of this therapeutic protein.

1. Introduction

Haemophilia A is an X chromosome-linked disorder characterised by insufficient production of functional Factor VIII (FVIII) (Larner, 1987). FVIII contains the domain structure A1-A2-B-A3-C1-C2 (Fay, 1993; Foster and Zimmerman, 1989), which is defined based on internal homology. The A-domains are homologous to the copper-binding protein ceruloplasmin (Kane and Davie, 1986; Church et al., 1984) and the two C domains are homologous to the C domains of Factor V and lactadherin (Stubbs et al., 1990; Hvarregaard et al., 1996). The B-domain does not show homology to other known domains. However, its size and complex structure including 19 out of totally 25 potential N-linked glycosylations sites present in FVIII is similar to the structure of the B-domain in Factor V.

FVIII circulates in plasma as an inactive heterodimer, non-covalently bound to von Willebrand factor (vWF). Only after activation of FVIII with thrombin is the binding to vWF abolished (Hamer et al., 1987). The thrombin catalyzed activation cleavage causes release of the residues 1649–1689, also known as the small acidic a3 domain, and the release of the a3 domain is responsible for the abrogation of the binding between FVIII and vWF (Hill-Eubanks et al., 1989; Lollar et al., 1988). Thrombin activation also results in cleavage at residues 372 and 740. Hereafter, FVIII interacts with phosphatidylserine (PS) on activated platelets, which results in an acceleration of the coagulation cascade (Gilbert and Drinkwater, 1993). In plasma, vWF is necessary in order to maintain a normal level of FVIII. This is evident from patients with vWF disease, who have low levels of vWF, or non-functional vWF, resulting in low levels of FVIII in plasma (Tuddenham et al., 1982).

Previously, FVIII has been purified from human plasma and used as therapeutic agent. This was later found to have severe risks due to the contamination of the plasma-derived FVIII with blood borne infectious vira such as Hepatitis C and HIV. Increasing knowledge in the area of recombinant protein production technology allowed for recombinant production of FVIII (rFVIII) *in vitro* with mammalian cells as production hosts. It has been necessary to use mammalian cells as production host, due to the extensive post-translational modifications rFVIII undergoes intracellularly. However, mammalian cells have specific demands for nutrition

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and cultivation, which combined with the very low yield of rFVIII production makes it difficult and very costly to produce rFVIII.

Insight into the FVIII structure and function has made it possible to design rFVIII variants, which significantly increase the yield in the rFVIII production. First, it was found that the B-domain was dispensable for rFVIII activity and a B-domain deleted (BDD) rFVIII was produced and is on the market today (Toole et al., 1986). Later, it was found that mutation of a single amino acid in rFVIII (Phe309Ser) reduced interaction with the endoplasmatic reticulum resident chaperone BiP and resulted in a several fold increase in secretion of rFVIII (Swaroop et al., 1997). Recently, the importance of the B-domain and the N-linked glycosylations, which are found extensively in this domain, were investigated (Miao et al., 2004). From the same study it was observed that by re-introducing a part of the N-terminal B-domain, the expression level was raised significantly. Optimisation of the rFVIII yield from the cell cultivation medium has until now been done by altering the rFVIII structure. Further, the requirement for addition of vWF to the medium has been demonstrated (Kaufman et al., 1989; Adamson, 1994). vWF is believed to stabilise the interaction between the heavy chain (HC) and the light chain (LC) of rFVIII (Kaufman et al., 1988) and also provides protection from proteolytic degradation.

Interaction of rFVIII with the plasma membrane of the production cells in serum free medium has been suggested, and it was proposed that this interaction led to degradation of the membrane bound rFVIII (Adamson, 1994). Later, it was demonstrated that rFVIII could be released from the membrane surface by subjecting the cells to a substance with high ionic strength (Winge, 2006). Thus, it seems that the rFVIII attached to the membrane was not degraded as first suggested, but was sequestered on the membrane resulting in a reduced amount of rFVIII protein in the culture medium.

In the present study we have searched for possible agents that could be used to hinder or limit the interaction between rFVIII and the cell surface of the production host cell. Annexin V was discovered due to its anticoagulant effect (Reutelingsperger et al., 1985) and it is assumed that annexin V competes with blood coagulation factors for the exposed phosphatidylserine on the platelet surface. It was found that sheets of clustered annexin V molecules are present on surfaces expressing phosphatidylserine and therefore it was suggested that this is the mechanism by which rFVIII interaction to the phosphatidylserine is hindered (Andree et al., 1992). We investigated whether this compound would also be able to reduce the membrane binding of rFVIII to the production cells. We used medium containing 0.5 M NaCl to release membrane bound rFVIII and an iodinated rFVIII competition assay to measure the membrane bound fraction of rFVIII. We demonstrate that the yield of rFVIII in the culture medium can be increased by adding vWF. Furthermore, the addition of annexin V and OPLS resulted in significant reduction in the membrane bound fraction of rFVIII.

The adopted nomenclature in this manuscript is that the BDD-FVIII N0 construct (see plasmid preparation under Section 2) is simply referred to as rFVIII. When data from other papers are discussed, rFVIII refer to the BDD-FVIII variant employed in the relevant paper.

2. Materials and methods

2.1. Materials

a-MEM medium, 10% dialysed FBS, pCR®-Blunt II-TOPO® vector, 293fectin, freeStyle HEK-293-F cells, freeStyle medium, DMEM, 4–12% Bis–Tris acrylamide gels, PVDF membranes (Invitrogen, Paisley, UK), MTX (Wyeth, Taplow, UK), HiSpeed plasmid Maxi kit (Qiagen, Hilden, Germany), restriction enzymes (New England Biolabs, Hitchin, UK), pTT5 vector (National Research Council, Montreal, Canada), F8C-EIA FVIII ELISA kit (Affinity Biologicals, ON, Canada), coamatic Factor VIII activity test (Chromogenix, Milan, Italy), SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Thermo Scientific, Cramlington, UK), anti-human Factor VIII affinity purified polyclonal sheep IgG antibody (Cedarlane, Ontario, Canada), rabbit anti Sheep HPR-conjugated antibody, HRP conjugated rabbit anti mouse IgG antibody (DAKO, Glostrup, Denmark), phosphatidylserine, phosphatidylcholine (Sigma–Aldrich Corp., St. Louis, MO), ESH8 mouse anti human FVIII:LC antibody (American Diagnostica Inc., Stamford, CT), Polysorp microtititer plates (NUNC, Roskilde, Denmark), TMBOne (Kem-En-Tec, Taastrup, Denmark).

2.2. Plasmid preparation

A partial B-domain deleted FVIII (N0) construct containing a 21 aa B-domain linker consisting of 10 aa from the N-terminal part of the B-domain (SFSQNSRHPS) and 11 aa from the C-terminal part of the B-domain (QNPPVLKRHQR) was made by fusing the FVIII heavy-chain with the FVIII light-chain. The 2300 bp heavy-chain was PCR-amplified by the primers 5′ primer (agtcgacgccaccatggaaatagagctctccac) and 3′ primer AgeI″ (gaccggtggattctgggagaagcttcttggttcaatggcattgtttttac) using WT FVIII as template. The 2100 bp light-chain was PCR-amplified by the 5′ primer AgeI″ (caccggtcttgaaacgccatcaacgggagatcactcgtactactcttc) and 3′ primer (aggatcctcagtagaggtcctgtgcctcg) using WT FVIII as template. An AgeI, ccaccggtc, restriction site was introduced via the primer to facilitate future subcloning. The two FVIII fragments were cloned into pCR-bluntII (Invitrogen) vector and sequence analysed. The 2100 bp BamHI-BamHI fragment of FVIII-light-chain was cloned into pMPSV-HE and the direction was checked by digestion with restriction enzymes. Second, the 2300 bp SalI-AgeI fragment of FVIII-heavy-chain was cloned into the former construct.

The following primers were used to amplify FVIII(del-a3) without the acidic a3 domain (EITRTTLQSDQEEIDYDDTISVEMKKEDFDIYDEDENQSPR). The upstream primer (aaaacccaccggtcttgaaacgccatccccgcagctttcaaaagaaaacacg) was placed in the beginning of the A3 domain and the downstream primer (cccaatggcatgctgcaactatttaaatcacagcc) was placed in the C2 domain containing the *Sph*I site. The restriction digested DNA fragment was ligated into the N0-BDD vector creating FVIII(del-a3).

A FVIII construct (N1) containing 28 aa of the B-domain was cloned by restriction digestion of the full length FVIII followed by subcloning. The DNA fragment was gel purified and restriction digested with *Nru*I and *Age*I. The vector N0 was cut with the same enzymes and the DNA fragment ligated into this vector. The resulting plasmid was controlled by sequence analysis and restriction digestion.

2.3. Cell cultures stably expressing rFVIII

Experiments were performed using Chinese hamster ovary (CHO) cells derived from a dihydrofolate reductase minus ($dhfr^-$) CHO DUKX B11 host. dhfr-CHO-DUKX-B11 cells were transfected with the rFVIII (N1) plasmid using Fugene 6 (Roche) following the manufactures instructions in a-MEM medium (Gibco) supplemented with ribonucleosides and deoxyribonucleosides and 10% dialyzed fetal bovine serum (FBS). Two days after transfection the cells were transferred to a-MEM medium (Gibco) without ribonucleosides and deoxyribonucleosides but with 10% dialysed FBS. The transfected clones were selected for stable integration for 15 days. This was followed by a stepwise amplification with MTX up to a concentration of 1 μM MTX. Cells were adapted to grow in serum free medium by gradually reducing the concentration of serum in the medium. Cells were cultivated as suspension cells in serum free