Novel therapies for haemophilia A – the role of the von Willebrand factor chaperone.

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The FVIII-VWF complex

Factor VIII (FVIII) is a plasma sialoglycoprotein that plays a critical role in maintaining normal haemostasis. Patients with severe haemophilia A have markedly reduced plasma FVIII levels and thus typically demonstrate a significant bleeding phenotype. Accumulating data suggests that plasma factor VIII is predominantly derived from biosynthesis within sinusoidal and endothelial cells (EC), particularly in the liver and lung [1]. FVIII is initially synthesized as an inactive 2332 amino acid polypeptide, composed of three distinct domain types: A, B and C (domain structure A1-a1-A2-a2-B-a3-A3-C1-C2). Prior to secretion, this single-chain FVIII undergoes complex post-translational modification that includes significant glycosylation, sulfation, and limited intracellular proteolytic processing. Consequently, plasma FVIII circulates as a heterodimeric protein consisting of a heavy chain (A1-a1-A2-a2-B) and a light chain (a3-A3-C1-C2), held together through a metal-ion dependent interaction.

In plasma, the majority of heterodimeric FVIII circulates in high affinity complex with von Willebrand factor (VWF) [1]. A number of recent studies have provided important insights into the biological basis underpinning the FVIII-VWF interaction. In particular, several discrete sites within the FVIII light chain (including the N-terminal acidic *a3*-region and the C-terminal C1-C2 domains) have been implicated in modulating its binding to VWF. In contrast, the FVIII-binding site has been mapped to the D'D3 domains of VWF. Under normal physiological conditions, approximately 95% of FVIII is bound to VWF, whilst the remaining 5% circulates in free form. This pool of free FVIII is maintained as a result of ongoing dissociation of FVIII from VWF in the peripheral circulation. Interaction with VWF serves to stabilize heterodimeric FVIII, and also protects FVIII from premature proteolytic degradation and clearance. Thus, in the absence of its VWF carrier, the plasma half-life of free FVIII is markedly reduced from 12 hours to 2 hours [1]. This is exemplified in patients with type 2N VWD who have D'D3 mutations that impair normal FVIII binding. As a result FVIII clearance is significantly enhanced in these patients and plasma FVIII levels are reduced leading to a bleeding tendency.

FVIII-VWF complex and in vivo clearance

In addition to advances in our understanding of the FVIII-VWF interaction, recent studies have also provided important insights into the biological mechanisms through which free FVIII and the FVIII-VWF complex are cleared from the plasma [1]. In particular, in vitro studies have demonstrated that VWF and FVIII can bind and be endocytosed by a number of

different cell types, including macrophages, hepatocytes, liver sinusoidal endothelial cells (LSEC), hepatocytes and dendritic cells (DCs). A number of specific cellular scavenger receptors have been implicated in regulating VWF-FVIII binding. These include the low-density lipoprotein receptor-related protein-1 (LRP1) which is expressed on macrophages; the scavenger receptor class A member I (SR-A1; also known as SCARA1 or CD204) expressed on macrophages and DCs; and Stabilin-2 (STAB2) which is expressed on LSECs. Perhaps unsurprisingly given the fact that both FVIII and VWF are heavily gylcosylated, roles for a number of individual lectin receptors in regulating FVIII-VWF clearance have also been described [1]. These lectins include the asialoglycoprotein (ASGPR) or Ashwell-Morell receptor, the macrophage galactose-type lectin (MGL), Siglec-5 and CLEC4M. Current evidence suggests that most of these receptors can bind to both free FVIII and the FVIII-VWF complex. However, since the vast majority of plasma FVIII is circulating in high-affinity complex with VWF, it seems likely that most FVIII is cleared whilst coupled to VWF. Further studies will be required in order to elucidate the relative importance of different individual receptors in terms of both physiological and pathological FVIII clearance. Interestingly however, genome-wide association studies (GWAS) have reported significant associations between FVIII-VWF plasma levels and genes encoding a number of these putative clearance receptors [1]. Moreover, specific polymorphisms in some clearance receptors have also been associated with variation in plasma FVIII-VWF levels.

FVIII-VWF complex – clinical relevance in the treatment of haemophilia A

Previous studies have demonstrated that the in vivo clearance of recombinant FVIII (rFVIIII) varies widely between different patients with haemophilia A (PWH), with the plasma half-life of rFVIII ranging between 6 and 29 hours. This inter-individual variation in rFVIII clearance has direct clinical significance. In particular, studies have demonstrated that for patients with severe haemophilia A on prophylaxis, increasing time per week with plasma FVIII less than 0.01 IU/ml is associated with significantly increased risk for bleeding episodes [2]. The biological mechanisms responsible for the variability in rFVIII clearance between different PWH have not been well defined. However, since the majority of FVIII is cleared in complex with VWF, it seems likely that inter-individual variations in endogenous VWF clearance rates play a key role. This hypothesis is supported by data from previous studies showing that rFVIII survival in patients with haemophilia A is significantly influenced by plasma VWF levels [2]. Consequently, FVIII half-life is significantly longer in patients with elevated VWF levels (e.g. patients with non-O blood groups) [2].

In addition to regulating the clearance of FVIII, accumulating evidence suggests that VWF-binding may also play a role in regulating FVIII immunogenicity (reviewed in Lai *et al*, Blood 2017) [3]. For example, in vitro data suggest that VWF protects FVIII endocytosis by DCs, and that VWF may also modulate DC presentation of FVIII peptides [3]. These observations are intriguing, given clinical data suggesting that the risk for inhibitor development may be higher in PWH treated with recombinant FVIII compared to those treated with plasma-derived FVIII products containing VWF [1,3].

FVIII-VWF complex and novel therapies for haemophilia A

Regular treatment with rFVIII prophylaxis significantly protects against spontaneous bleeding episodes in PWH [2]. Unfortunately however, since the circulatory half-life of FVIII in plasma is short, standard FVIII prophylaxis regimens necessitate regular intravenous rFVIII infusions to maintain therapeutic plasma FVIII levels. This dosing schedule impacts negatively upon patient quality of life, and also has important implications for patient compliance [2]. In an effort to improve treatment for PWH, different strategies have been utilized in an effort to develop novel rFVIII therapies with extended half-lives (EHL-FVIII). These strategies include site-specific PEGylation, glycoPEGylation and covalent coupling of rFVIII to the Fc domain of human IgG1 [1]. These protein-engineering strategies have proved effective in extending in vivo survival for a number of other coagulation glycoproteins (e.g. FIX and FVII) up to 5-fold. In striking contrast however, clinical studies with modified rFVIII molecules have demonstrated more moderate increases in half-life (approximately 1.5-fold) compared to that of wild type rFVIII [1]. Although the underlying mechanism(s) have not been defined, it seems likely that this limited prolongation of variant rFVIII half-life reflects that fact that the majority of FVIII actually circulates in plasma in complex with VWF. Consequently, the molecular mechanisms through which FVIII and VWF interact in vivo is of critical importance when it comes to developing novel EHL-FVIII therapies.

Previous studies mapped the FVIII binding region to the D'D3 domains of VWF. Furthermore, Yee *et al* recently reported that a VWF D'D3 polypeptide containing only residues S764 – P1247 of full length VWF could bind FVIII with high affinity in vitro, and effectively prolong endogenous FVIII survival in VWF-deficient mice following hydrodynamic expression [4]. In addition, extending the circulatory survival of the VWF D'D3 fragment by Fc fusion (VWF D'D3-Fc) also resulted in a significant prolongation in FVIII half-life in VWF-deficient mice [4]. In marked contrast however, the VWF D'D3-Fc

carrier molecule was unable to significantly prolong the half-life of co-infused FVIII in FVIII-deficient mice. The molecular mechanisms underlying this discrepancy have not been fully defined, but are likely to relate in large part to competition between the VWF D'D3-Fc carrier molecule and endogenous full length VWF for FVIII binding. In an effort to circumvent this problem of FVIII dissociating from the long-acting D'D3 carrier molecule, studies are ongoing with a novel recombinant FVIIIFc-VWF-XTEN (rFVIIIFc-VWF-XTEN) fusion model in which four different proteins have been used together (single chain B-domain deleted FVIII (BDD-FVIII), VWF D'D3, XTEN (an unstructured biodegradable protein polymer designed to increase the half-lives of therapeutic peptides and proteins) and the Fc region of IgG1) [1].

Nanobody manipulation of FVIII-VWF complex equilibrium

In a recent paper, Muczynski and colleagues propose another innovative strategy that might be utilized to develop an EHL-rFVIII therapy [5]. This approach exploits our knowledge of the biochemistry underpinning the FVIII-VWF interaction and involves the use of a novel FVIII-nanobody fusion protein that can form an ultra-stable complex with endogenous wild type VWF. Consequently, the proportion of free FVIII (which has a short half life of only 2 hours) in the plasma is markedly reduced. In brief, the authors first developed a novel llama-derived nanobody that recognized an epitope located within the D'D3 domain of VWF. Subsequently, two copies of this nanobody were used to replace the B domain residues (Gln744-Arg1648) in a novel single chain FVIII fusion protein termed FVIII-KB013BV. Importantly, the FVIII fusion protein retained its normal thrombin-activation sites at Arg372, Arg740 and Arg 1689 respectively such that thrombin activation caused nanobody insert release and allowed FVIIIa to assemble into the membrane-bound extrinsic Xase complex. Consequently, full cofactor activity was observed for FVIII-KB013BV in both one-stage clotting and chromogenic assays. Kinetic binding experiments confirmed that the FVIII-KB013BV fusion protein was 25-fold more efficient in binding to VWF compared to BDD-VWF [5]. In keeping with these in vitro data, the mean residence time of FVIII-KB013BV following intravenous infusion was significantly increased in FVIII-deficient mice, despite the fact that these mice have normal endogenous plasma VWF levels. Overall the half-life of FVIII-KB013BV was increased approximately 2-fold compared to BDD-FVIII. Moreover, the FVIII-KB013BV retained functional activity and in contrast to BDD-FVIII was still able to significantly attenuate blood loss in a tail clip assay performed 24h after infusion. All together, these novel findings suggest that despite the fact that only a minority of FVIII

circulates free in normal plasma, nevertheless improved FVIII-VWF binding can be targeted in order to significantly enhance FVIII survival in vivo.

The importance of free FVIII compared to VWF-bound FVIII in terms of their relative immunogenicity in PWH remains poorly defined. Nevertheless, as previously discussed there is some data to suggest that VWF may modulate FVIII uptake and presentation by DCs [1,3]. Consequently, Muczynski *et al* also examined the potential immune response generated towards FVIII-KB013BV in FVIII-deficient mice following repeated dose administrations [5]. Interestingly, they observed a significantly attenuated response. Anti-FVIII antibodies were observed in only 12.5% of mice treated with FVIII-KB013BV compared to 87.5% of mice treated with BDD-FVIII. Although preliminary in nature, these data suggest that reduction in the levels of free FVIII may not only provide a novel mechanism to prolong FVIII circulatory half-life, but also serve to attenuate rFVIII immunogenicity. Given the significant morbidity and mortality that continue to be associated with FVIIII inhibitor formation in PWH, the potential clinical significance of these findings is readily apparent.

In conclusion, interaction with its VWF chaperone plays a critical role in modulating many aspects of FVIII biology. As our understanding of the molecular mechanisms involved in the FVIII-VWF interaction continue to develop, it seems likely that additional opportunities to develop novel therapeutic EHL-FVIII products with reduced immunogenicity are likely to emerge.

Addendum

S.A. and J.S.OD both were involved in writing and revising the manuscript

Disclosure of Conflict of Interests

S.A. and J.S.OD have no relevant conflicts of interest.

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