Galectin-1 and Galectin-3 Constitute Novel-Binding Partners for Factor VIII

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Objective—Recent studies have demonstrated that galectin-1 (Gal-1) and galectin-3 (Gal-3) can bind von Willebrand factor and directly modulate von Willebrand factor-dependent early thrombus formation in vivo. Because the glycans expressed on human factor VIII (FVIII) are similar to those of von Willebrand factor, we investigated whether galectins might also bind and modulate the activity of FVIII.

Approach and Results—Immunosorbant assays and surface plasmon resonance analysis confirmed that Gal-1 and Gal-3 bound purified FVIII with high affinity. Exoglycosidase removal of FVIII N-linked glycans significantly reduced binding to both Gal-1 and Gal-3. Moreover, combined removal of both the N- and O-glycans of FVIII further attenuated Gal-3 binding. Notably, specific digestion of FVIII high-mannose glycans at N239 and N2118 significantly impaired FVIII affinity for Gal-1. Importantly Gal-1, but not Gal-3, bound to free FVIII in the plasma milieu, and significantly inhibited FVIII functional activity. Interestingly, commercial recombinant FVIII (rFVIII) concentrates are manufactured in different cell lines and differ in their glycosylation profiles. Although the biological mechanism has not been defined, recent studies in previously untreated patients with severe hemophilia A reported significant differences in inhibitor development associated with different rFVIII products. Interestingly, Gal-1 and Gal-3 both displayed enhanced affinity for BHK–rFVIII compared with CHO–rFVIII. Furthermore, binding of Gal-1 and Gal-3 to BDD–FVIII was markedly reduced compared with full-length rFVIII.

Conclusions—We have identified Gal-1 and Gal-3 as novel-binding partners for human FVIII and demonstrated that Gal-1 binding can influence the procoagulant activity of FVIII.


Key Words: factor VIII ■ galectins ■ glycosylation ■ lectins ■ plasma

Factor VIII (FVIII) is a plasma sialoglycoprotein that plays an essential role in hemostasis by acting as a cofactor for the serine protease factor IXa.2, 13 Plasma FVIII is primarily derived from endothelial cells3,4 and is initially synthesized as a 2332 amino acid polypeptide, composed of 3 distinct domain types: A, B, and C (domain structure A1–a1–A2–a2–B–a3–A3–C1–C2).5–7 Before secretion, this single-chain FVIII polypeptide undergoes complex post-translational modification, including significant glycosylation and limited intracellular proteolytic processing.8 In plasma, FVIII binds to von Willebrand factor (VWF) with high affinity \((K_a=0.2–0.5 \text{ nmol/L})^{9,10}\)

On the basis of primary amino acid sequence, each FVIII molecule contains 21 N-glycosylation sites, 17 of which are located within the B-domain.8 As shown in Figure 1, a further 4 potential N-linked glycan sites are located in both the heavy (2 in the A1 domain) and light chains (1 in the A3 and 1 in the C1 domain) of FVIII. In addition, the FVIII B domain also contains at least 7 O-linked glycans.11,12 The N-linked oligosaccharide chains of human plasma-derived and recombinant FVIII (rFVIII) have been characterized.13,14 Bi-antennary, tri-antennary, and tetra-antennary complex-type chains, together with high-mannose–type sugars were identified.13,14 Unusually, the complex N-linked oligosaccharides of plasma FVIII were also shown to express covalently linked ABO(H) blood group antigenic determinants.13 This expression of ABO(H) antigenic determinants on plasma FVIII and VWF has direct clinical significance because ABO blood
group is a critical determinant of plasma FVIII–VWF levels. Blood group O subjects have significantly lower plasma levels (≈25%) of both FVIII:C and VWF:Ag compared with non-O individuals. The mechanism through which ABO blood group determines plasma FVIII–VWF levels remains poorly defined, but is likely to be mediated at least in part, through variation in FVIII–VWF clearance.

Previous studies have shown that FVIII glycans have important biological effects. In particular, FVIII B-domain glycans play a critical role in regulating intracellular trafficking. First, B-domain glycans modulate FVIII binding to the protein chaperones calnexin and calreticulin within the endoplasmic reticulum. Second, complex N-linked oligosaccharides clustered within the B domain also interact with the endoplasmic reticulum–Golgi transport receptor LMAN1–MCFD2 complex. Furthermore, recent studies have shown that FVIII glycans outside the B domain are also of functional importance. For example, Dasgupta et al demonstrated that specific mannose-terminating glycan chains on FVIII influence dendritic cell uptake mediated through the macrophage mannose receptor (CD206) and subsequent presentation to CD4+ T cells. Importantly, these findings suggest that glycan expression may influence the immunogenic properties of FVIII.

Although the importance of FVIII glycans is well established, the molecular mechanisms through which carbohydrate expression influences FVIII biology remain poorly understood. However, FVIII has been shown to bind several specific lectins, including the asialoglycoprotein receptor and the mannose receptor CD206,23 The galectins are a family of soluble lectins characterized by specific affinity for β-galactosides. Galectin-1 (Gal-1) and galectin-3 (Gal-3) are abundantly expressed in endothelial cells. Significant plasma galectin concentrations have also been reported (1–10 μg/mL). Interestingly, Saint-Lu et al recently reported that Gal-1 and Gal-3 circulate in normal plasma in complex with VWF. Furthermore, galectin binding significantly influenced the formation of platelet-decorated VWF strings on EC surfaces. In addition, a novel role for Gal-3 in the pathophysiology of venous thrombosis has also been recently described. Given that the carbohydrate structures expressed on human FVIII are similar to those on VWF, we hypothesized that Gal-1 and Gal-3 could constitute novel-binding partners for FVIII.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.
Results

Gal-1 and Gal-3 Bind to Human FVIII

The complex N- and O-linked glycan structures expressed on human VWF and FVIII share many similarities, including terminal sialylation and ABO(H) blood group expression (Figure 1). Consequently, given that Gal-1 and Gal-3 have been shown to bind to VWF, we hypothesized that these galectins could constitute novel-binding partners for FVIII. The potential interaction between FVIII and galectins was initially investigated in experiments using purified proteins. In immunosorbant plate-binding assays, we observed that purified recombinant Gal-1 and Gal-3 both displayed dose-dependent and saturable binding to immobilized pd-FVIII (Figure 2A and 2B). In contrast, minimal binding of Gal-1 and Gal-3 to BSA-coated control wells was observed. To exclude the possibility that VWF present in the plasma-derived purified FVIII preparation might be confounding results, plate-binding assays were repeated using rFVIII expressed in CHO cells. Previous studies have demonstrated that the glycans expressed on this rFVIII are similar to those on pd-FVIII. In keeping with our previous findings, both Gal-1 and Gal-3 displayed dose-dependent and saturable binding to immobilized rFVIII (Figure 3A and 3B). Interestingly, some differences in galectin binding to pd-FVIII compared with rFVIII were observed. This binding variation likely reflects in large part the significant differences in glycosylation that exist between pd-FVIII and rFVIII. The binding of Gal-1 and Gal-3 to rFVIII was further assessed using surface plasmon resonance. These surface plasmon resonance studies confirmed dose-dependent and reversible binding of both Gal-1 (KD 0.47 nmol/L) and Gal-3 (KD 0.12 nmol/L) to rFVIII (Figure 3C and 3D). Collectively, these findings demonstrate that Gal-1 and Gal-3 constitute novel-binding partners for human FVIII. Moreover, with the exception of VWF, it is interesting that the apparent affinities of Gal-1 and Gal-3 for FVIII are much higher than those previously reported for other galectin ligands.

Specific N- and O-Linked Glycans on FVIII Modulate Individual Galectin Interactions

Previous studies demonstrated that galectin interactions with VWF were inhibited in a dose- and time-dependent manner in the presence of lactose. Similarly, in preliminary studies we observed that lactose also markedly attenuated the binding of both Gal-1 and Gal-3 to FVIII (data not shown). In contrast, equal concentrations of sucrose had no significant effect on galectin–FVIII interactions. To further define the role of specific FVIII glycan structures in regulating galectin interaction, FVIII glycans were modified using a series of specific exoglycosidases. After glycosidase treatments, residual FVIII glycan expression was characterized using modified lectin ELISAs as previously described. Digestion with Peptide N-glycosidase F (PNGase F) resulted in a marked reduction in FVIII N-linked glycan expression detected by Concanavalin A (Figure 4A). Furthermore, combined treatment with PNGase F and O-glycosidase resulted in an additional significant decrease in FVIII O-linked T antigen expression detected by Jacalin (Figure 4B). Importantly, removal of the N-linked glycan of FVIII (PNG–FVIII) markedly attenuated binding to Gal-1 (8.6±1%; P<0.001; Figure 4C). Although PNGase F digestion also significantly reduced FVIII binding to Gal-3 (residual binding 30.2±2%; P<0.001), the effect was less marked than that observed for Gal-1 (Figure 4D). Furthermore, combined removal of both the N- and O-glycans of FVIII (PNG–Ogly–FVIII) further attenuated Gal-3 binding (16.5±2%; P<0.05; Figure 4D). In contrast however, Gal-1 binding to PNG–Ogly–FVIII was not significantly different to that of PNG–FVIII. Together, these data demonstrate that FVIII interacts with galectins in a glycan-dependent manner. However, although Gal-1 interacts predominantly with the N-linked glycans of FVIII, both the N- and O-linked glycans of FVIII modulate interaction with Gal-3.

Commercial FVIII Concentrates Differ in Their Galectin-Binding Properties

Although a variety of commercial rFVIII products have been developed, these concentrates have been expressed in different cell lines (including CHO and BHK) and consequently express significantly different glycosylation profiles.
example, Gal-α(1,3)Gal structures have been identified on ≈3% of BHK-expressed rFVIII.13 Given the critical importance of FVIII glycans in regulating its interaction with galectins, galectin binding to various commercial rFVIII concentrates was examined. Interestingly, both Gal-1 and Gal-3 displayed increased binding to BHK–rFVIII compared with CHO–rFVIII (107.3±2% and 128.5±3%, respectively; P<0.05; Figure 4E and 4F). This finding is consistent with previous data suggesting that α1 to 3 galactosylation that is expressed on BHK-derived rFVIII constitutes a preferential galectin-binding ligand.31 Local clustering of complex glycan chains has been shown to critically regulate galectin-binding interactions.32 Importantly, 17 of the 21 potential N-glycosylation sites, and all of the O-linked glycans sites of FVIII are clustered within the B domain. To examine the importance of the glycan-rich B domain in modulating galectin binding, we further investigated Gal-1 and Gal-3 interactions with BDD–FVIII. In keeping with the loss of the majority of its glycans, binding of Gal-1 and Gal-3 to BDD–FVIII was markedly reduced compared with full-length rFVIII (42.2±1% and 19.6±1%, respectively; P<0.0001; Figure 4E and 4F).

Gal-1 Binding to FVIII Is Modulated by High-Mannose N-Linked Glycans

Interestingly, we observed that Gal-1 retained significant binding to BDD–FVIII. FVIII has 4 non–B-domain glycans located at N41 and N239 in the A1 domain, N1810 in the A3 domain, and N2118 in the C1 domain (Figure 1). Of these, N239 and N2118 have been identified as high-mannose–type oligosaccharides.11,14 To investigate a role for these glycans in modulating Gal-1 binding, BDD–FVIII was digested with either endoglycosidase H to selectively remove FVIII high-mannose structures at N239 and N2118 (endoglycosidase H BDD–FVIII), or with PNGase F to cleave all remaining N-glycans (PNG BDD–FVIII). Removal of N-linked glycans from BDD–FVIII was confirmed using the lectin Concanavalin A (Figure 5A). Moreover, specific removal of high-mannose chains was detected using the mannose-specific lectin, Galanthus nivalis (Figure 5B). Importantly, endoglycosidase H BDD–FVIII displayed significantly attenuated Gal-1 binding compared with unmodified BDD–FVIII (33.0±4.9% versus 100.0±11%, respectively, P<0.001; Figure 5C). Moreover, the reduced binding of endoglycosidase H BDD–FVIII and PNG BDD–FVIII were similar in magnitude, suggesting that
the high-mannose glycans at N239 and N2118 are of particular importance in regulating Gal-1 interaction. This observation is important because these FVIII high-mannose glycans have been implicated in regulating FVIII immunogenicity.21

Gal-1 Can Bind to Free FVIII Within the Plasma Milieu

Having demonstrated that Gal-1 and Gal-3 both bind with high affinity to purified FVIII in vitro, we further investigated whether these galectins could also interact with FVIII in plasma. Because Gal-1 and Gal-3 both bind VWF, we examined galectin–FVIII binding in VWF-deficient plasma. As illustrated in Figure 6A, beads coated with recombinant Gal-1 or Gal-3, or untreated control beads were incubated with plasma obtained from a patient with type 3 von Willebrand disease. After incubation and washing, the beads were analyzed by Western blotting using antigalectin or anti-FVIII heavy chain antibodies. We observed that Gal-1–coated beads precipitated FVIII from human VWF-deficient plasma (Figure 6A). In contrast however, no FVIII was precipitated by either the Gal-3–coated beads or the control untreated control beads. Similarly, beads coated with recombinant Gal-1 (but not Gal-3 or untreated control beads) also precipitated FVIII from plasma obtained from VWF-deficient mice (data not shown). The observation that Gal-1 can bind to free FVIII in the plasma, whereas Gal-3 cannot is interesting, given that their relative in vitro binding affinities were similar (Figures 2 and 3).

Binding of Gal-1 Significantly Attenuates FVIII Activity

Given that activated FVIII (FVIIIa) exists as a B-domainless heterotrimer, our data suggest that Gal-1 may also bind to FVIIIa. Consequently we further investigated whether galectin binding influences FVIII activity. After incubation with increasing concentrations of either Gal-1 or Gal-3 (0.25–16 μmol/L), the procoagulant activity of the galectin-bound rFVIII–CHO in FVIII-deficient plasma was assessed using a 1-stage APTT clotting assay. Interestingly, addition of Gal-1 resulted in a significant and dose-dependent attenuation in rFVIII activity (Figure 6B). For example, in the presence of 2 μmol/L Gal-1, the APTT was prolonged from 26.6±3 s to 54.7±0.3 s; *P<0.005. The half maximal inhibitory concentration (IC50) for Gal-1 was calculated at 0.35±0.02 μmol/L. In contrast, Gal-3 had no significant effect on FVIII coagulant activity.
activity. FVIII activity in the presence or absence of galectins was further investigated using a purified FXa generation assay (Figure 6C). Interestingly, we observed that Gal-1 binding again significantly reduced FVIII activity in a dose-dependent manner. In contrast, even at high concentrations, Gal-3 had no significant effect on FVIII activity.

Discussion
Glycan expression on human FVIII has been shown to influence important aspects of FVIII biology. For example, glycan determinants clustered within the B domain of FVIII play a critical role in facilitating intracellular trafficking. Although previous studies have demonstrated that FVIII glycans bind to specific lectins, including asialoglycoprotein receptor and CD206, nevertheless the molecular mechanisms through which carbohydrate determinants on FVIII influence its function remain poorly understood. The galectins are a highly conserved family of carbohydrate-binding proteins that demonstrate affinity for β-galactoside structures. In total, 11 different human galectins have been described. These galectins are expressed in many different cell types (including endothelial cells, platelets, and macrophages) and are also present in normal plasma. In this study, we demonstrate for the first time that FVIII can interact with at least 2 members of the galectin family (Gal-1 and Gal-3, respectively). Although a large number of putative galectin-binding partners have previously been described, our data demonstrate that the apparent affinities of Gal-1 and Gal-3 for pd-FVIII (in the range 0.12–0.47 nm/L) are unusually high compared with those reported for other galectin-glycoprotein interactions. This observation is likely because of the clustered complex branching carbohydrate structures expressed on human FVIII. In addition, glycan microarray studies have shown that both Gal-1 and Gal-3 can bind to ABO blood group determinants, which are also expressed on FVIII glycans. Interestingly, VWF is the only other human glycoprotein that displays levels of galectin-affinity comparable with those of FVIII.

In addition to containing carbohydrate recognition domains that modulate interaction with glycan structures, galectins have also been shown to engage in direct protein–protein interactions. However, our findings demonstrate that the interaction of both Gal-1 and Gal-3 with FVIII is predominantly glycan mediated. Interestingly, the specific FVIII glycan determinants involved in modulating binding with Gal-1 and Gal-3 display important differences. Although both the N- and O-linked carbohydrate chains of FVIII influence Gal-3 binding, we found that the N-linked glycans of FVIII were mainly responsible for Gal-1 interaction. Moreover, in contrast to the critical role of the glycan-rich B domain in modulating Gal-3 binding, our data demonstrate that high-mannose N-linked glycans located outside the B domain are important in modulating Gal-1 interaction. This observation is important since Dasgupta et al previously demonstrated that these high-mannose glycans play a critical role in regulating FVIII immunogenicity by influencing dendritic cell FVIII uptake via the macrophage mannose receptor (CD206). Consequently, blocking the CD206 receptor, or enzymatic digestion of the high-mannose glycans of FVIII, were both shown to result in reduced dendritic cell endocytosis and attenuated T-cell activation. We postulate that Gal-1 binding may thus modulate FVIII antigenicity by binding to these high-mannose glycan determinants. In addition, previous studies have shown that Gal-1 also plays other direct roles in modulating both innate and adaptive immune responses through different signaling pathways, including regulation of T-cell survival and cytokine secretion. Furthermore, recent studies of previously untreated patients with severe hemophilia A reported significant differences in inhibitor development between different rFVIII products. Our findings clearly demonstrate that different commercial rFVIII concentrates display significant differences in their relative galectin affinities. Further studies will be required to determine whether differences in rFVIII–galectin interactions may be important with respect to modulating endogenous immune responses in patients with severe hemophilia A.

Collectively, our findings clearly demonstrate that Gal-1 and Gal-3 both bind to purified FVIII in vitro with high affinity. To further define the physiological relevance of this observation, galectin-FVIII binding was also investigated within the plasma milieu. We observed that Gal-1–coated beads...
precipitated FVIII from VWF-deficient plasma. In contrast, no FVIII was precipitated by either Gal-3–coated beads or control untreated beads. These data are interesting given that the binding affinities of Gal-1 and Gal-3 for purified FVIII in vitro were similar. However, previous studies have suggested that Gal-3 is able to bind to a much broader range of plasma ligands compared with Gal-1 (including α2-macroglobulin and transferrin). Consequently, it seems likely that these other glycoproteins may outcompete free FVIII for Gal-3 binding. Nevertheless, Saint-Lu et al recently reported that Gal-3 circulates in complex with VWF in normal plasma. Because the majority of FVIII also circulates in complex with VWF, it remains unclear whether Gal-3 oligomers bound to VWF may also interact with tethered FVIII.

It is well recognized that in the absence of VWF binding, plasma FVIII half-life is markedly reduced (from 12 to 2 hours). Although the biological basis underlying the rapid clearance of free FVIII remains poorly understood, recent studies suggest that hepatic and splenic macrophages play key roles in mediating FVIII clearance. Furthermore, a specific role for the macrophage lipoprotein receptor in mediating the clearance of free FVIII has been reported. In addition, previous studies have shown that macrophage lipoprotein receptor can form heterologous functional complexes with several other cellular receptors, including β2-integrins. In this context, it is interesting that our data demonstrate that Gal-1, which is also expressed on macrophages, can bind to free FVIII and, therefore, influence procoagulant activity. Interestingly, after incubation with Gal-1, the procoagulant activity of rFVIII was significantly attenuated in a dose-dependent manner in both a 1-stage clotting assay and in a FXa generation assay. In contrast, Gal-3 had no effect on FVIII activity. These data are consistent with our previous observations that (1) the major Gal-3–binding site is located within the FVIII B domain and (2) Gal-3 does not bind to FVIII within the plasma milieu. Despite the high apparent affinity of Gal-1 for FVIII, relatively high concentrations were needed to reduce FVIII activity. This finding likely relates, in part, to the significantly reduced affinity of Gal-1 on macrophages or other cell types may be involved in modulating the in vivo clearance of FVIII.

Because Gal-1 can bind to BDD–FVIII, and also to free FVIII in plasma, we hypothesized that Gal-1 may be able to bind to FVIIIa and, therefore, influence procoagulant activity. Interestingly, after incubation with Gal-1, the procoagulant activity of rFVIII was significantly attenuated in a dose-dependent manner in both a 1-stage clotting assay and in a FXa generation assay. In contrast, Gal-3 had no significant effect on FVIII coagulant activity. These data are consistent with our previous observations that (1) the major Gal-3–binding site is located within the FVIII B domain and (2) Gal-3 does not bind to FVIII within the plasma milieu. Despite the high apparent affinity of Gal-1 for FVIII, relatively high concentrations were needed to reduce FVIII activity. This finding likely relates, in part, to the significantly reduced affinity of Gal-1 for FVIIIa, in which the glycan-rich B domain has been cleaved. Nevertheless, collectively our data demonstrate that Gal-1 is capable of adhering to
VIIIa through specific interaction with high-mannose glycans located within the A1 domain (Asn239) of the heavy chain and C1 domain (Asn2118) of the light chain. The mechanism(s) through which this Gal-1 binding then serves to attenuate VIIIa functioning as a cofactor for factor IXa within the intrinsic FXase complex remains to be determined. However, the concentration of Gal-1 needed to inhibit VIIIa procoagulant activity (IC 50–0.3 μmol/L) is significantly lower than those previously reported to be associated with platelet activation (3–12 μmol/L).48 Moreover, accumulating evidence suggests that local concentrations of Gal-1 may be markedly elevated at sites of vascular injury. Platelets express significant amounts of Gal-1 that is secreted after platelet activation.48 Furthermore, Gal-1 is also expressed within EC, where it is stored together with VWF within Weibel Palade bodies and secreted after EC activation.76,28,34 In addition, markedly elevated systemic plasma galectin levels have also been demonstrated in association with many pathological conditions, including acute inflammatory disorders and metastatic malignancy.77,30,31

Interestingly, a series of elegant recent studies have identified novel roles for Gal-1, Gal-3, and Gal-3–binding protein in the pathogenesis of venous thrombosis.27,52,53 Moreover, several different molecular mechanisms have been implicated in this galectin-modulated link between coagulation and inflammation, including upregulation of P selectin, platelet activation, and enhanced microparticle formation.52 Additional studies will be required to determine whether galectin interactions with FVIII and VWF may also be important in this context.

In conclusion, this study shows that Gal-1 and Gal-3 both bind to purified human FVIII in a dose-dependent manner with high affinity. Moreover, these individual galectin interactions are modulated by specific N- and O-linked glycan determinants expressed on FVIII. Our data further demonstrate that different commercial rFVIII concentrates display significant differences in their respective galectin-binding affinities. Given the important role played by Gal-1 in regulating inflammatory responses, and recent studies reporting significant differences in antigenicity between different rFVIII clotting factor concentrates, we postulate that variation in binding to galectins and other lectins may be important in modulating differences in immunogenicity between different rFVIII products.

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Disclosures
J.S. O’Donnell has served on the speaker’s bureau for Baxter, Bayer, Novo Nordisk, Boehringer Ingelheim, Levo Pharma, and Octapharma. He has also served on the advisory boards of Baxter, Bayer, Octapharma CSL Behring, Daiichi Sankyo, Boehringer Ingelheim, and Pfizer. J.S. O’Donnell has also received research grant funding awards from Baxter, Bayer, Pfizer, and Novo Nordisk.

References
We have identified a novel interaction between human galectins, specifically galectin-1 and galectin-3, with human coagulation glycoprotein factor VIII (FVIII). Moreover, galectin binding is modulated by N- and O-linked glycans expressed on FVIII. We have demonstrated a specific role for FVIII high-mannose structures, in the A1 and C1 domain, in regulating galectin-1 binding. In contrast, FVIII glycans within the B domain play an important role in mediating galectin-3 binding. Critically, galectin-1, but not galectin-3, bound to free FVIII in human plasma and significantly inhibited FVIII procoagulant activity in vitro. Interestingly, galectin-1 and galectin-3 display distinct binding preferences for various commercial FVIII concentrates. Recent studies have reported significant differences in antigenicity between a number of recombinant FVIII commercial concentrates. Given the important role defined for galectins in influencing inflammatory responses, we postulate that variation in lectin binding may be important in modulating differences in immunogenicity between recombinant FVIII products.

**Significance**

We have identified a novel interaction between human galectins, specifically galectin-1 and galectin-3, with human coagulation glycoprotein factor VIII (FVIII). Moreover, galectin binding is modulated by N- and O-linked glycans expressed on FVIII. We have demonstrated a specific role for FVIII high-mannose structures, in the A1 and C1 domain, in regulating galectin-1 binding. In contrast, FVIII glycans within the B domain play an important role in mediating galectin-3 binding. Critically, galectin-1, but not galectin-3, bound to free FVIII in human plasma and significantly inhibited FVIII procoagulant activity in vitro. Interestingly, galectin-1 and galectin-3 display distinct binding preferences for various commercial FVIII concentrates. Recent studies have reported significant differences in antigenicity between a number of recombinant FVIII commercial concentrates. Given the important role defined for galectins in influencing inflammatory responses, we postulate that variation in lectin binding may be important in modulating differences in immunogenicity between recombinant FVIII products.
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MATERIALS AND METHODS

Materials

A complete description of the reagents used in this study is included in the supplemental Material. In brief, VWF-free plasma-derived (pd-)FVIII was from Biotest® and was kindly supplied by Dr. David Lillicrap, Queen's University Kingston, Canada. Recombinant FVIII concentrates studied included; full-length FVIII expressed in Chinese Hamster Ovary (rFVIII-CHO) cell line (Advate® - Baxalta); full-length FVIII expressed in Baby Hamster Kidney cells (rFVIII-BHK), (Kogenate® - Bayer); and B-domain deleted FVIII (BDD-FVIII) manufactured in CHO cell lines (Refacto® - Pfizer). Recombinant His-tagged galectin-1 and -3 were expressed in E.coli and purified using a nickel chloride charged Hi-Trap chelating column (GE Healthcare).

FVIII-galectin binding studies

FVIII (plasma-derived, recombinant or glycan-modified) was immobilized onto a 96-well PolySorb microtiter plate (Nunc, UK), at concentrations of 2 - 0.15nM in 50mM sodium carbonate buffer (pH 9.6) overnight at 4°C. The wells were washed three times in PBS-T and blocked for 2 hours using 0.5% (w/v) Polyvinylpyrrolidone (PVP) solution in PBS-T. Gal-1 (150nM) or Gal-3 (500nM) were incubated on the plate for 2 hours at 37°C. Bound Gal-1/-3 were detected using specific mouse monoclonal antibodies, anti-galectin-1 (Santa Cruz Biotechnology, USA) and anti-galectin-3 (R&D Systems, MN, USA) and goat anti-mouse HRP-conjugated antibody (Santa Cruz Biotechnology, USA). After washing, HRP-activity was measured by addition of o-phenylenediamine substrate (Sigma-Aldrich, Ireland) and stopped with 1M H2SO4. Absorbance was measured at 450nm. FVIII-galectin binding kinetics
were further investigated determined using surface plasmon resonance (SPR) analysis on a BIACore 3000 system. For SPR analysis, rFVIII was covalently immobilized onto flow cell two of a CM5 sensor chip using an Amine Coupling Kit (GE Healthcare, UK) in 10mM sodium acetate buffer pH 5.0. Flow cell one was activated, capped and used a reference cell. For binding analysis, increasing concentrations of purified Gal-1 or Gal-3 were passed over the surface of the chip at a flow rate of 30 μL/min in HEPES buffer (10mM HEPES, 3mM EDTA, 150mM NaCl and 0.005% (v/v) Tween 20, pH 7.4 with a contact time of 120 seconds. Dissociation was allowed for 10 minutes and the chip surface was regenerated with 10mM NaOH. The resulting sensograms were fit globally to a heterogeneous ligand model using BIAevaluation software (GE Healthcare, UK).

**Modification of rFVIII glycan structures**

To modify endogenous FVIII glycan structures, rFVIII (50µg/ml) was incubated overnight at 37°C under non-reducing conditions with specific exoglycosidases including; 500U Peptide N Glycosidase F (PNGase F) (from Flavobacterium meningosepticum; New England Biolabs, UK), 500U Endoglycosidase H (from Streptomyces picatus; New England BioLabs, UK) and 1.5U O-glycosidase (from Streptococcus pneumonia; Sigma, Ireland). All digestions were performed in triplicate. Following each glycosidase digestion, changes in FVIII carbohydrate expression were assessed with the use of specific lectin ELISAs. Glycosidase-treated rFVIII and control unmodified rFVIII (0.1-2µg/ml) were immobilised onto MaxiSorb microtiter wells (Nunc, UK) in phosphate-buffered saline (PBS) pH 7.4 for 2 hours at 37°C. Non-specific binding was blocked with Protein-Free Blocking Buffer (Thermo Fisher Scientific, UK) for 1 hour at 37°C. Biotinylated lectins including Concanavalin
*A, Jacalin* and *Galanthus Nivalis* (Vector Laboratories, UK) were incubated at 1µg/ml in PBS for 1 hour at 37°C. Lectin binding was detected streptavidin–horseradish peroxidase (R&D Systems, UK) and subsequent incubation with substrate 3,3’,5,5’-Tetramethylbenzidine (TMB; R&D Systems, UK). Absorbance was read at 450nm and lectin binding was expressed as a percentage of control unmodified rFVIII.

**Galectin – FVIII binding in plasma**

Briefly, 100 µg of purified His-tagged recombinant Gal-1 or Gal-3 were incubated with 200µl of cobalt chelate beads (Talon, Clontech, France) in binding buffer (50mM sodium phosphate, 300mM sodium chloride and 20 mM imidazole pH 8.0) for 1 hour at room temperature. After the galectin-coated beads had been washed thoroughly in PBS-T, 20µl of beads were incubated with 0.5mL of VWD type 3 human plasma for 1 hour at room temperature. Following incubation, the beads were separated from plasma by centrifugation as before and washed in triplicate with PBS-T. Proteins bound to the beads were stripped by heating in the presence of 200mM DTT. The samples were then analysed by SDS-PAGE immunoblotting using rabbit polyclonal anti-FVIII heavy chain antibody (0.4µg/mL) (Santa Cruz Biotechnology, USA), and rabbit polyclonal anti-Gal-1 (0.25µg/mL) (Zymed, Invitrogen, USA) or rabbit polyclonal anti-Gal-3 (0.7µg/mL) (Abcam Cambridge, UK). All antibodies were diluted in 3% BSA in PBS-T and incubated for 1 hour at 37°C. Blots were washed thoroughly in PBS-T before applying secondary HRP-conjugated antibody (goat anti-rabbit IgG-HRP; Santa Cruz Biotechnology, USA).
FVIII Activity

FVIII activity was determined using a one-stage APTT assay. Briefly, 1nM rFVIII was incubated in the presence or absence of increasing concentrations of either Gal-1 or Gal-3 (0.25-16µM). Samples were subsequently mixed with FVIII-deficient plasma (Langanbach, Ireland) at 37°C for 3 minutes and APTT clotting time measured in the presence of kaolin, cephalin (C.K. Prest. Stago Beijing, China), and 20mM CaCl₂ using Amelung KC4 Micro Clinical Coagulation Analyzer (Amelung, Trinity Biotech, Ireland). Alternatively, FVIII activity with/without increasing concentrations of Gal-1 was measured following the conversion of FX to FXa using a commercial Chromogenix™ assay (Instrumentation Laboratory, MA, USA) as per manufacturer’s instructions.

Data presentation and statistical analysis

All experimental data and statistical analysis were performed using the GraphPad Prism program (Graphpad Prism version 5.0 for Windows; GraphPad Software, Inc. San Diego, CA). Data were expressed as mean values ± standard error of the mean (SEM). To assess statistical differences, data were analysed using Student’s unpaired 2-tailed t test. For all statistical tests, P values <0.05 were considered significant.