Identification of Galectin-1 and Galectin-3 as Novel Partners for Von Willebrand Factor

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Objective—Although von Willebrand factor (VWF) is a heavily glycosylated protein, its potential to associate with glycan-binding proteins is poorly investigated. Here, we explored its interaction with the glycan-binding proteins galectin-1 and galectin-3.

Methods and Results—Immunofluorescence analysis using Duolink proximity ligation assays revealed that VWF colocalizes with galectin-1 and galectin-3 in endothelial cells, both before and after stimulation of endothelial cells. Moreover, galectin-1 was found along the typical VWF bundles that are released by endothelial cells. Galectin-1 and galectin-3 could be coprecipitated with VWF from plasma in immunoprecipitation assays, whereas plasma levels of galectin-1 and galectin-3 were significantly reduced in VWF-deficient mice. Binding studies using purified proteins confirmed that VWF could directly interact with both galectins, predominantly via its N-linked glycans. In search of the physiological relevance of the VWF-galectin interaction, we found that inhibition of galectins in in vitro perfusion assays was associated with increased VWF-platelet string formation, a phenomenon that was reproduced in galectin-1/galectin-3 double-deficient mice. These mice were also characterized by a more rapid formation of initial thrombi following ferric chloride–induced injury.

Conclusion—We have identified galectin-1 and galectin-3 as novel partners for VWF, and these proteins may modulate VWF-mediated thrombus formation. (Arterioscler Thromb Vasc Biol. 2012;32:894-901.)

Key Words: endothelium ■ hemostasis ■ platelets ■ galectin ■ von Willebrand factor

Von Willebrand factor (VWF) is an adhesive protein that is critical to the recruitment of platelets in response to vessel injury. The majority of the circulating VWF molecules are produced in the endothelial cells, where VWF is synthesized as a single prepropolypeptide chain. Following signal peptide removal, the polypeptides are assembled into C-terminal linked pro-VWF dimers. Further processing includes proteolytic removal of the propeptide and multimerization of the molecule by intermolecular N-terminal cysteine bonding, generating a pool of differentially sized multimers that may contain more than 50 subunits. An important portion of the newly synthesized VWF multimers is directed to endothelial storage organelles, Weibel-Palade (WP) bodies. VWF is obligatory for the formation of WP bodies, which are indeed absent in endothelial cells isolated from VWF-deficient mice or dogs. These storage organelles also contain other proteins besides VWF, including P-selectin, osteoprotegerin, CD63, and interleukin-8. Some of these proteins directly interact with VWF, which may facilitate their uptake into the WP bodies. For other residents of the storage granules, the uptake may be the result of a more random inclusion process. Following endothelial stimulation, the content of the WP bodies is released into the circulation, allowing the first encounter between VWF and circulating platelets. Indeed, VWF multimers assemble into twisted bundles and networks that form long strings along the endothelial surface. These VWF strings are able to catch platelets, and these platelet-decorated strings can be visualized via microscopy in vitro and in vivo.

During its synthesis, VWF is also subject to extensive glycosylation. The mature VWF subunit contains 12 sites for N-linked glycosylation and 10 sites for O-linked glycosylation. The main N-linked glycan element has been identified to be a complex-type biantennary core-fucosylated structure (60% of all glycans, corresponding to 7 or 8 per monomer), with the majority being sialylated. An additional 13% of the N-linked glycan structures (1 or 2 per monomer) are characterized by the presence of ABO blood group determinants. The O-linked glycome is represented by the sialylated T-antigens (galactose-(β1-3)-N-acetyl-galactosamine), which constitute...
70% to 90% (7–9 per monomer) of all O-linked structures.15,16 About a quarter of these T-antigens contain disialyl structures, indicating that the terminal galactose or N-acetylgalactosamine residues are capped with 2 rather than 1 sialic acid.16

The carbohydrate structures on VWF play an important role in the various steps of the VWF life-cycle. First, N-linked carbohydrate structures are needed for optimal folding and multimerization of the protein.11,17 Second, sialic acid residues capping the glycan structures limit spontaneous binding to platelets18,19 and render the molecule more sensitive to proteolysis by ADAMTS13.20 ABO-structures are known for their role in the regulation of VWF plasma levels. Individuals with blood group O have 20% to 30% lower plasma levels compared with those with non-O blood groups.21 Finally, we have recently found that also O-linked glycans contribute to the regulation of VWF plasma levels.22

Despite the importance of carbohydrate structures in the life-cycle of VWF, little is known about the interaction between VWF and carbohydrate-binding proteins. The human proteome consists of several families specialized in the interaction with glycan structures, including the galectin family. Galectins represent an evolutionarily highly conserved family of proteins that interact with β-galactoside residues.23 The human family members consists of 11 members, some of which are (like VWF) expressed in endothelial cells.24 In particular, galectin-1 (Gal-1) and galectin-3 (Gal-3) are being abundantly expressed in the endothelium.24–26 Moreover, glycan array analysis has revealed that both Gal-1 and Gal-3 are able to recognize carbohydrate structures that are present on the VWF molecule.27

Here, we explored the possibility that Gal-1 and Gal-3 are able to interact with VWF. Our study shows that Gal-1 and Gal-3 colocalize with VWF in endothelial cells and remain associated following stimulated secretion. Furthermore, plasma levels of Gal-1 and Gal-3 are significantly reduced in VWF-deficient mice and can be normalized following hepatic VWF expression. Finally, we observed that the absence of Gal-1 and Gal-3 is associated with more efficient formation of platelet-decorated VWF strings along the endothelial surface and with enhanced formation of arterial thrombi. In conclusion, our data identify Gal-1 and Gal-3 as novel partners for VWF, the physiological relevance of which seems related to the regulation of VWF-mediated thrombus formation.

**Materials and Methods**

An extensive description of materials and methods can be found in the online-only Data Supplement. A brief summary is given below.

**Mice**

Three mouse strains on a C57B6/J background were used throughout the study: wild-type mice, VWF-deficient mice,28 and Gal-1/Gal-3 double-deficient mice (Jackson Laboratory, Bar Harbor, ME).

**Duolink Proximity Ligation Assay**

Fixed human umbilical vein endothelial cells (HUVECs) were stained with a pool of mouse monoclonal anti-VWF antibodies and 1 of the following goat antibodies: anti-histone-H3, anti-osteoprotegerin, anti-Gal-1, or anti-Gal-3. The Duolink proximity ligation assay (Duolink-PLA) assay was then performed as instructed by the manufacturer (Olink Bioscience, Uppsala, Sweden). Each discrete red spot represents a single VWF-protein complex (radius <40 nm).

**Immunoprecipitation Assays**

Protein G–coupled magnetic beads (Dynabeads-Protein G, Invitrogen) were coated with antibodies against Gal-1, Gal-3, VWF or control IgG as instructed. Following incubation with plasma, beads were washed and incubated with SDS-PAGE sample buffer. Subsequently, samples were analyzed via Western blotting using anti-VWF or anti-Gal-3 antibodies. Immunoreactive bands were visualized using chemiluminescence.

**VWF-Galectin Binding Studies**

Two types of binding studies were performed. First, immunosorbert studies were performed, in which microtiter wells were coated with Gal-1, Gal-3, or bovine serum albumin. Wells were then incubated with various concentrations of VWF, and bound VWF was detected using peroxidase-labeled polyclonal VWF antibodies. Second, binding was assessed using biolayer interferometry (Octet-QK equipment, Fortebio). Gal-1 and Gal-3 were coupled to amine-reactive biosensors and incubated with various concentrations of VWF. Association and dissociation were both followed for 10 minutes.

**In Vivo Perfusion Assays**

Washed human platelets were perfused over HUVEC-containing coverslips at a shear rate of 300 s⁻¹ as described.29

**In Vivo Thrombosis and String Formation**

In vivo analysis of VWF-platelet string formation and ferric chloride-induced thrombosis was performed on mesenteric vessels that were prepared as described3,28 except that rhodamine-6G was used to label platelets fluorescently. VWF-platelet string formation was induced by the topical application of ionophore A23187. Ferric chloride-induced injury of mesenteric arterioles was induced by the application of a filter soaked in 7.5% (wt/vol) FeCl₃.

**Results**

Gal-1 and Gal-3 Are Associated With VWF in Endothelial Cells

It has previously been shown that endothelial cells express Gal-1 and Gal-3.24–26 This led us to hypothesize that perhaps part of the Gal-1 and Gal-3 proteins could colocalize with VWF, the majority of which is located in endothelial storage organelles, the WP bodies. To test for colocalization, we applied Duolink-PLA analysis, which detects proteins located within a radius of <40 nm. First, the validity of this approach was established by monitoring colocalization of VWF with the WP body coresident osteoprotegerin in HUVECs. As a negative control, we stained HUVECs for VWF in combination with nuclear protein histone-H3. Whereas background staining was observed for VWF/histone-H3, distinct red spots were detected for VWF/osteoprotegerin (Figure 1 in the online-only Data Supplement). In quantitative terms, 3.5-fold more fluorescence intensity was detected for VWF/osteoprotegerin than for VWF/histone-H3 (P = 0.011; Figure IC in the online-only Data Supplement). We could also detect a similar pattern of red spots throughout HUVECs when applying Duolink-PLA analysis to VWF/Gal-1 and VWF/Gal-3 (Figure 1). This indicates that both galectins localize within 40 nm of VWF, suggesting that part of the Gal-1 and Gal-3 population may colocalize with VWF in endothelial cells. Colocalization was also examined in phorbol 12-myristate 13-acetate (PMA)-stimulated HUVECs. As expected, PMA
PMA stimulation (Figure 1), suggesting that colocalization was observed for VWF/Gal-1 and VWF/Gal-3 following histone-H3 (Figure 1). In contrast, a strong fluorescent signal stimulation did not affect the lack of staining for VWF/Gal-1 (Figure 1F). Indeed, additional analysis using classical immunofluorescence confocal-microscopy demonstrated the presence of Gal-1 along the typical VWF bundles (Figure 1F). Taken together, these experiments indicate that both Gal-1 and Gal-3 colocalize with VWF in endothelial cells both before and after endothelial stimulation.

**Gal-1 and Gal-3 Remain Associated With VWF Following Secretion**

Given the colocalization of VWF and galectins, we determined whether Gal-1 or Gal-3 remains associated with VWF in plasma. Normal human plasma was incubated with anti-VWF, anti-galectin, or control antibodies coated on magnetic protein G beads. Precipitated beads were then analyzed by Western blotting using anti-galectin or anti-VWF antibodies. VWF could be detected in samples precipitated using anti-Gal-1 or anti-VWF antibodies but not in control precipitations (Figure 2A). Furthermore, Gal-3 was detected in samples precipitated using anti-VWF or anti-Gal-3 antibodies but not in control precipitations (Figure 2B). Apparently, both Gal-1 and Gal-3 circulate in plasma in complex with VWF. The association between VWF and these galectins in plasma was further investigated by assessing Gal-1 and Gal-3 levels in wild-type and VWF-deficient mice, which were matched for sex and age. Levels of Gal-1 were 4.1±1.0 and 1.5±0.6 ng/mL in wild-type (n=11) and VWF-deficient mice (n=8; P<0.0001), respectively (Figure 2C). For Gal-3, levels were 16.4±5.4 and 10.8±2.4 ng/mL in wild-type (n=11) and VWF-deficient mice (n=8; P=0.025), respectively (Figure 2D). Of interest, levels of both Gal-1 and Gal-3 could be corrected following hydrodynamic gene transfer of VWF, which results in normalization of VWF levels in VWF-deficient mice (not shown). These data indicate that VWF contributes to the regulation of plasma levels of Gal-1 and Gal-3.

**Gal-1 and Gal-3 Directly Interact With VWF**

To further characterize the potential interaction between VWF and galectins, a series of binding studies using purified proteins was performed. First, different concentrations of VWF (0–50 μg/mL) were added to wells coated with Gal-1, Gal-3, or bovine serum albumin, and bound VWF was detected using anti-VWF antibodies. No binding of VWF to bovine serum albumin–coated control wells was observed (Figure 3). In contrast, VWF displayed dose-dependent and saturable binding to Gal-1 and Gal-3 (half-maximal binding 21.4±4.1 μg/mL and 4.7±0.4 μg/mL, respectively). In complementary studies, we also observed binding of biotinylated Gal-1 or Gal-3 to immobilized VWF (not shown). In a second series of experiments, the interaction was tested via biolayer-interferometry analysis using Octet-QK-equipment. Also using this approach, VWF was able to interact with immobilized Gal-1 or Gal-3 in a dose-dependent and reversible manner (Figure 3A and 3B, insets). We then investigated whether the interaction of both galectins with VWF involves carbohydrate structures. First, binding of VWF to immobi-
lized Gal-1 or Gal-3 was inhibited in a dose- and time-dependent manner in the presence of lactose but not in the presence of mannose (not shown). Second, VWF was preincubated in the absence or presence of N-glycosidases endoF2/endoF3 and added to immobilized Gal-1 or Gal-3. Bound VWF was subsequently detected using polyclonal anti-VWF antibodies, the reactivity of which was not affected by deglycosylation of VWF (not shown). Binding of VWF to Gal-1 and Gal-3 was remarkably reduced following incubation of VWF with N-glycosidases (Figure 3). In conclusion, VWF interacts directly with Gal-1 and Gal-3 in a glycan-dependent manner.

Galectins Modulate VWF-Platelet Interactions at the Endothelial Cell Surface

In search of a physiological role of the VWF-galectin interaction, we started from the observation that VWF and Gal-1 and Gal-3 are associated within the endothelial cells and remain in complex following secretion.10 We therefore investigated whether galectins affect VWF-platelet string formation. Platelets were perfused over a monolayer of HUVECs at a shear rate of 300 s⁻¹. On exposure to laminar flow, HUVECs secreted long strings of VWF multimers to which platelets adhered. In the absence of any additives, 2.1±0.9 (n=12) strings per microscopic field were observed (Figure 4A). A similar number of strings per field (1.9±0.8; n=12) was found in the presence of glucose (30 mmol/L), a sugar that leaves galectin function unaffected (Figure 4A). Surprisingly, the number of strings per field was significantly increased when platelets were perfused in the presence of lactose (5.3±3.4; n=12; P<0.0003), which blocks galectins (Figure 4A). Apart from the number of strings per field, we also observed that the number of platelets per string was affected by the presence of lactose. To quantify this phenomenon, the number of platelet present per micrometer of string was analyzed. A similar number of platelets per micrometer of string was found for both control conditions (0.23±0.05 and 0.22±0.05 platelets/μm of string for no additives and glucose, respectively; Figure 4B). In contrast, increased platelet coverage was obtained in the presence of lactose (0.52±0.14 platelets/μm of string; P<0.0001; Figure 4B). These data suggest that under galectin-blocking conditions, VWF multimer assembly at the endothelial surface is modulated in that more strings are being formed and more platelets can bind per string.

Galectins Contribute to the Early Stage of Thrombus Formation

Because lactose-mediated inhibition is not specific to Gal-1 and Gal-3 but may also affect other galectins and other proteins, we further addressed the role of galectins in VWF multimer assembly using mice that were deficient for both Gal-1 and Gal-3. Preliminary analysis revealed that plasma levels of VWF and factor VIII were similar in Gal-1/Gal-3-deficient mice and wild-type control mice (not shown). The
formation of platelet-rich strings in vivo was monitored via real-time intravital microscopy in the mesenteric venules of wild-type and Gal-1/Gal-3−/− mice. Endothelial cells in the venules were stimulated via application of ionophore A23187, and the number of strings was counted during a 10-minute observation period. Fewer strings were formed in wild-type mice compared with Gal1/Gal3−/− mice, 14.2±5.6 versus 23.8±5.9 (P=0.022; Figure 5A). This confirms that the absence of Gal-1 and Gal-3 is associated with more efficient VWF-string formation. In another series of experiments, we then examined how the absence of Gal-1 and Gal-3 would affect arterial thrombus formation in a ferric chloride–induced thrombosis model. Two parameters were examined: time to first thrombus (≥30 μm) and time to first occlusion. No differences in time to first occlusion were observed between wild-type mice and Gal-1/Gal-3−/− double-deficient mice (not shown). In contrast, thrombi of ≥30 μm were formed more rapidly in Gal1/Gal3−/− mice compared with wild-type mice (6.4±1.1 versus 8.3±2.0 minutes; P=0.021; Figure 5B). Apparently, more efficient string formation in Gal-1/Gal-3-deficient mice favors early thrombus formation in these mice. Taken together, our data are compatible with a model in which Gal-1 and Gal-3 act as negative modulators of VWF-mediated thrombus formation at the endothelial surface.

**Discussion**

VWF is a heavily glycosylated protein, and its glycans contribute to the various stages of its life cycle, including biosynthesis, function, and clearance. It is surprising, therefore, that little is known about the potential of VWF to associate with glycan-binding proteins. In the current study, we have obtained evidence that VWF interacts efficiently with 2 members of the family of glycan-binding galectins, ie, Gal-1 and Gal-3. A first hint for an interaction between VWF and galectin-1 (Gal-1) and galectin-3 (Gal-3). Figure 3 shows Glycan-dependent interactions between von Willebrand factor (VWF) and galectin-1 (Gal-1) and galectin-3 (Gal-3). A and B, Microtiter wells coated (5 μg/mL) with Gal-1 (filled circles, A), Gal-3 (filled circles, B) or bovine serum albumin (open circles) were incubated with various concentrations of VWF (0–50 μg/mL in A, 0–15 μg/mL in B). After washing, horseradish peroxidase–labeled polyclonal VWF antibodies (diluted 1:1000) were added to the wells, and bound antibodies were detected by measuring horseradish peroxidase activity using o-phenylenediamine as a substrate. Plotted is the absorbance vs the VWF concentration added. Insets A and B, Gal-1 (5 μg/mL) with Gal-1 (G) or Gal-3 (6 μg/mL) (D). Bound VWF was detected as described for A and B. Plotted is the absorbance observed for VWF incubated in the absence or presence of EndoF2/F3. Data represent the mean±SD of 3 independent experiments.

Figure 4. Increased von Willebrand factor (VWF)–platelet string formation on inhibition of galectins. Platelets were perfused over human umbilical vein endothelial cells (HUVECs) at a shear of 300 s−1. Snapshots were taken after 2 minutes of perfusion in the absence or presence of glucose (100 mmol/L) or lactose (100 mmol/L). Microscopic fields were analyzed for the number of VWF–platelet strings per field (A) and the number of platelets per μm of string (B). Data represent the mean±SD of 12 different fields obtained during 2 perfusions using different batches of HUVECs. Statistical analysis was performed using 1-way ANOVA/Dunnett multiple comparison test. **P<0.001, ***P<0.0001. Insets show representative images of perfusions in the absence (I) or presence (II) of lactose.
The positive staining for VWF/Gal-1 and VWF/Gal-3 indicates that both galectins are closely located to VWF in the endothelial cells (Figure 1). It has previously been reported that Gal-1 and Gal-3 are present in the cytoplasm and at the cell surface of endothelial cells. The possibility cannot be excluded that some of the VWF multimers are located outside the WP bodies and interact with Gal-1 and Gal-3 in the cytoplasm. Alternatively, given the dimensions of the VWF-containing WP bodies, it seems conceivable that part of the Gal-1 and Gal-3 population coexists with VWF in the WP bodies. Indeed, another report on the expression of Gal-1 and Gal-3 in endothelial cells revealed a granule-like staining pattern for both proteins. The potential uptake of Gal-1 and Gal-3 into the WP bodies may be a random process, as has been described for a number of cytokine-residents of the WP bodies. On the other hand, our binding experiments demonstrate that VWF can interact directly with Gal-1 and Gal-3 (Figure 3). As such, VWF may actively target both galectins to the WP bodies, similar to what has been described for P-selectin and osteoprotegerin.

It should be noted that PMA-mediated stimulation of HUVECs resulted in an apparent stronger fluorescent Duolink-PLA signal (2–4 fold in quantitative terms; Figure 1). This increased complex formation seemed specific, as it was not observed for VWF/histone-H3 (Figure 1). It seems conceivable that this increased complex formation represents an artifact, as the release of VWF from the WP bodies may promote the accessibility of the protein for the detecting antibodies. Another interesting observation from our immunofluorescence experiments relates to the formation of string-like patterns following PMA-stimulation (Figure 1). It is well known that VWF assembles into large bundles following stimulated secretion. Our data suggests that Gal-1 is present along these strings, following VWF during its release from the endothelial cells.

Besides being in complex in endothelial cells, our immunoprecipitation assays also point to VWF being in complex with Gal-1 and Gal-3 in plasma (Figure 2). In addition, a significant decrease in Gal-1 and Gal-3 plasma levels was observed in mice deficient for VWF (Figure 2). Several scenarios could explain this link between VWF and galectin plasma levels. First, the presence of VWF could promote the synthesis/storage and secretion of Gal-1 and Gal-3, independently of eventual complex formation. Second, complex formation between VWF and these galectins may promote their circulatory life span. In this respect, it is tempting to speculate on a similarity between Gal-1 and Gal-3 versus coagulation factor VIII, a protein for which VWF functions as a carrier protein in plasma.

First, the 50- to 100-fold molar excess of VWF is similar for factor VIII, Gal-1, and Gal-3, indicating that only part of the VWF subunits is decorated with these proteins. Second, plasma concentrations of factor VIII, Gal-1, and Gal-3 are all markedly reduced in the absence of VWF, suggesting that VWF is important to stabilize plasma levels of each of these proteins. Additional clearance studies of Gal-1 and Gal-3 in VWF-deficient mice are needed to provide more insight in this issue. Nevertheless, our findings merit additional studies to the correlation between plasma levels of VWF and both galectins in normal subjects and patient groups that are characterized by abnormal levels (increased or decreased) of VWF.

Plasma levels of Gal-1 and Gal-3 could be normalized following hydrodynamic gene transfer of VWF in these VWF-deficient mice (not shown). In this approach, VWF is expressed in hepatocytes, cells that do not seem to express Gal-1 and Gal-3 under normal conditions. This could suggest that VWF secreted by the hepatocytes is able to bind and stabilize Gal-1 and Gal-3 in the circulation. Alternatively, the possibility cannot be excluded that VWF expression induces expression of both galectins, thereby reinforcing their plasma concentrations.

Using various experimental approaches, we demonstrated that VWF is able to directly interact with Gal-1 and Gal-3. Interestingly, the apparent affinities are in the range of 20 to 80 nmol/L, which seems higher than usual for these glycan-binding proteins. One potential explanation is that VWF not only carries at least 12 N-linked glycans per subunit, but these
subunits are also covalently linked into molecular structures that can contain >20 of such subunits. This multimerization may generate a clustered glycan surface, similar to that at the surface of cells. Binding of Gal-1 and Gal-3 to VWF could be inhibited in the presence of the galectin-antagonist lactose or via the removal of VWF-glycans via incubation with endoF2/F3 glycosidases (Figure 3). Apparently, VWF-galectin complex formation is mediated via protein-glycans interactions, predominantly involving N-linked glycans. This preference for N-linked glycans is compatible with the poor binding of Gal-1 and Gal-3 to sialylated O-linked glycans as found using glycan microarrays. In contrast, both Gal-1 and Gal-3 are able to interact with ABO-blood group determinants, which are present on the VWF molecule (1–2 per subunit). To this end, it is of interest that Gal-1 reacts similarly with A-, B-, and O-structures, whereas Gal-3 displays preferential binding to A- and B-structures. It is possible that these differences are reflected via a blood group–dependent VWF/Gal-3 ratio in human plasma.

In search of physiological relevance, we have tested the effect of Gal-1 and Gal-3 on the interaction between VWF and some of its ligands, glycoprotein-Ib and collagen. However, in these studies in which we used purified proteins, no significant inhibition or enhanced binding was observed (not shown), suggesting that Gal-1 and Gal-3 leave the interaction between VWF and its ligands glycoprotein-Ib or collagen unaffected. In contrast, we did observe a significant effect via lactose-dependent inhibition of galectin while perfusing platelets over endothelial cells in vitro (Figure 4). Of course, the observed lactose-based inhibition should not be considered conclusive evidence, because lactose may also inhibit other galectins. Nevertheless, it led us to investigate VWF-platelet string and VWF-dependent thrombus formation in vivo. Although the effect was modest from a quantitative point of view, a statistically significant increase in number of VWF-platelet strings was observed in Gal-1/Gal-3 double-deficient mice (Figure 5). Of note, the analysis of string formation was compromised by the short half-life of these strings because of ADAMTS13-mediated proteolysis of VWF at the endothelial surface. We did not observe differences in the life span of strings between wild-type and Gal-1/Gal-3 double-deficient mice, suggesting that galectin deficiency did not delay ADAMTS13-mediated proteolysis. However, more detailed studies using purified proteins would be needed to confirm this observation. In addition, crossings to obtain Gal-1/Gal-3/ADAMTS13 triple-deficient mice have been initiated.

In line with increased VWF-platelet string formation, small thrombi were also formed more rapidly in the absence of Gal-1 and Gal-3 (Figure 5). Again, the observed effect was modest but statistically significant. Interestingly, a similar time to first occlusion was observed, suggesting that the process from first thrombus to first occlusion proceeds slower in the absence of Gal-1 and Gal-3. Thrombus growth is strongly dependent on the function of integrin αIIbβ3. Indeed, it has recently been reported that Gal-1 contributes to platelet activation in an αIIbβ3-dependent manner. Given the role of αIIbβ3 in thrombus stabilization, it is not surprising, therefore, that our Gal-1/Gal-3 double-deficient mice were characterized by occlusive thrombi that were less stable compared with those of wild-type mice, as was apparent from a longer time to stable occlusion (not shown).

In conclusion, we have shown here that Gal-1 and Gal-3 should be considered novel partners for VWF and that this association is of physiological relevance. We would like to emphasize that our in vivo data are based on the use of double-deficient mice. Given the large overlap in the glycan structures that are being recognized by Gal-1 and Gal-3, we preferred to study in vivo thrombus formation in these double-deficient mice rather than in single-knockout mice to avoid redundancy. However, it has been reported that Gal-1 and Gal-3 may have differential effects on ligand binding. The possibility exists, therefore, that more pronounced effects on thrombus formation could be observed in single-knockout mice.

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Disclosures

None.

References


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Supplement Material I

Identification of Galectin-1 and Galectin-3 as novel partners for von Willebrand factor
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Expanded Material and Methods

Mice: Three mouse strains were used throughout the study, all of which were on a C57B6/J background: wild-type mice, VWF-deficient mice, and Galectin-1/Galectin-3 double-deficient mice (strain B6.Cg-Lgals3tm1Poi Lgals1tm1Rob/J) obtained from Jackson Laboratories (Ben Harbor, ME). Galectin-1/Galectin-3 double deficient mice did not display spontaneous abnormalities of hemostasis (thrombus formation or bleeding). Housing and experiments were carried out as recommended by the French regulations and Experimental Guidelines of the European Community. Animal experiments were approved by the Animal Care and Use Committee of Inserm (licence #B-94-043-13).

Proteins: VWF was purified from therapeutic VWF-concentrates via size-exclusion chromatography (110 U VWF antigen/mg protein; FVIII: <0.1 U/mg). Bovine serum albumin (BSA) was from Sigma (cat# A4503). Gal-1 was from R&D Systems (cat# 1152-GA/CF). Two sources were used for Gal-3: first, Gal-3 was obtained from R&D Systems (cat# 1154-GA/CF). Second, Gal-3 was purified from Gal-3 expressing E.coli-BL21. Briefly, IPTG-stimulated bacteria were harvested and sonicated. The supernatant was centrifuged, dialyzed against PBS/2 mM EDTA/4 mM mercaptoethanol and applied to a lactose-sepharose column. Lactose-binding Gal-3 was eluted from the column using PBS/2 mM EDTA/4 mM mercaptoethanol supplemented with 150 mM lactose. The eluate was dialyzed against PBS, and SDS-Page analysis confirmed the purity of Gal-3 to exceed 95%.

Cell culture: Two different sources of human umbilical vein endothelial cells (HUVECs) were used: HUVECs were obtained commercially (Promocell, Heidelberg, Germany) or were isolated from human umbilical cords as described, and cultured in endothelial cell basal medium-2 with supplements (Lonza, Walkersville, MD). Prior to experimentation, HUVECs were trypsinized and seeded on glass coverslips (4x10⁴ cells/well), allowing cells to grow to 60-80% confluency. HUVECs were stimulated via incubation with Phorbol 12-Myristate 13-Acetate (PMA; 20 ng/ml) for 30 min in serum-free medium containing 2 mM L-Glutamine/1% BSA.

Duolink-proximity ligation assay (Duolink-PLA): HUVECs were fixed with 4% paraformaldehyde for 30 min and then washed with PBS. After permeabilization for 5 min with 0.1 % Triton (Sigma, St Louis, MO), coverslips were blocked with PBS/3% ovalbumin for 30 min. Immuno-staining was performed by simultaneous incubation of a pool of anti-VWF monoclonal antibodies (10 µg/ml in PBS/3% ovalbumin) and one of the following polyclonal goat antibodies directed
against: histone-H3 (diluted 1:500; Abcam, Cambridge, UK), osteoprotegerin (1:10; R&D Systems, Minneapolis, Mn), Gal-1 (1:15; R&D Systems), or Gal-3 (1:30; R&D Systems). Dilutions of the antibodies were optimized in separate single staining experiments (not shown). The Duolink-PLA assay was subsequently performed as instructed (Olink Bioscience, Uppsala, Sweden). Briefly, this assay relies on the incubation with secondary antibodies coupled to complementary oligonucleotides that can hybridize when within a range of 40 nm. Following hybridization, this DNA template is amplified and highlighted using fluorescent-labeled oligonucleotides, generating one discrete red spot for each protein complex. The signals were visualized using an AxioImager A1 microscope (Carl Zeiss, Göttingen, Germany) using a Plan-Apochromat 63x-NA 1.4 objective. For quantification, 12 to 33 images of cells per condition were randomly collected, and analyzed using ImageJ-1.44 software (http://rsbweb.nih.gov/ij/index.html). Graphs represent the quantity of pixels covered by red fluorescence (uniformly thresholded between 170 and 255).

Confocal Immunofluorescence staining: HUVECs were prepared as described above and incubated with a pool of anti-VWF monoclonal antibodies (10 μg/ml in PBS/3% ovalbumin) and polyclonal goat anti-Gal-1 antibody (1:15; R&D Systems). After 30 min, coverslips were washed in PBS/0.1 % Tween-20 and incubated for 30 min with a donkey anti-mouse Alexa 488-conjugated antibody (1:1000; Invitrogen) and a donkey anti-goat Cy3-conjugated antibody (1:50; Abcam). Cells were then washed with PBS/0.1 % Tween-20 and distilled water, and mounted in fluorescent mounting medium (Dakocytomation, Glostrup, Denmark). Labeled cells were examined on an Axiovert 200M microscope using a Plan-Apochromat 63x-NA 1.4 - oil immersion objective and a Zeiss LSM510-meta confocal system (Carl Zeiss).

Immunoprecipitation assays: Protein G-coupled magnetic beads (50 μl Dynabeads Protein G, Invitrogen) were coated with 50 μg/ml of monoclonal rabbit anti-Gal-1 (EPR3205; Abcam), monoclonal rabbit anti-Gal-3 (EPR2774; Abcam), polyclonal rabbit anti-VWF (Dako), or non-specific polyclonal rabbit IgG as instructed by the manufacturer. Coated beads were then incubated overnight at 4°C with 1 ml of pooled normal human plasma (Cryopep, Montpellier, France), previously pre-cleared for nonspecific binding by a 2-h incubation at 4°C with rabbit-IgG conjugated magnetic beads. After three washes with PBS/0.1 % Tween-20, beads were resuspended in 60 μl PBS/0.1 % Tween-20, 20 μl of NuPAGE-LDS sample buffer and 0.8 μl of sample reducing agent (Invitrogen). After 10 min at 70°C, samples were separated using SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked for 1 hour with 5% milk in PBS/0.1 % Tween-20 and incubated overnight at 4°C with an in-house mixture of monoclonal anti-VWF antibodies (6 μg/ml) or polyclonal goat anti-Gal-3 antibodies (1:100; R&D systems). Bound antibodies were detected using horseradish-peroxidase (HRP)-conjugated anti-mouse IgG (1:2500, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-goat IgG (1:1000, R&D Systems). Immunoreactive bands were visualized using...
chemiluminescence (SuperSignal West Pico chemiluminescent substrate, Thermo Scientific, Rockford, IL).

**Gal-1 and Gal-3 levels in murine plasma:** Blood samples were collected at the retro-orbital sinus and centrifuged at 1500 g for 20 min to prepare plasma. Gal-1 and Gal-3 concentrations were measured using DuoSet ELISA kits for murine Gal-1 (DY1245; R&D Systems) and murine Gal-3 (DY1154; R&D Systems).

**VWF-galectin binding studies:** Micotiter wells were coated overnight with Gal-1, Gal-3 or BSA (5 µg/well). Wells were washed thrice with PBS/0.1% Tween-20 and blocked for 2 h by incubating with PBS/0.1 %TWEEN-20/3% BSA at 37°C. Subsequently, wells were washed and different concentrations of VWF were added (0-50 µg/ml) in PBS/0.1 %TWEEN-20/3% BSA. After 1 h at 37°C, non-bound VWF was removed, and bound VWF was detected using HRP-labeled polyclonal anti-VWF antibodies (1:1000, Dakocytomation), and HRP-activity was measured using o-phenylenediamine (OPD) as substrate. In some experiments, VWF was pretreated with glycosidases as follows. VWF (100 µg) was incubated in the absence or presence of EndoF2 and EndoF3 (Native protein deglycosylation kit, Sigma) as instructed for 72 h at 37°C. SDS-Page analysis of samples before and after treatment revealed a reduction in molecular size corresponding to a loss of glycans, while no degraded protein was detected.

**In vitro perfusion assays:** Washed human platelets were prepared as described³, and resuspended in Heps-Tyrode buffer (pH 7.2)/1% BSA in the absence or presence of glucose (2.8 %) or lactose (5.6 %). Platelet solutions (150x10³ platelets/µl) were subsequently perfused over HUVECs-containing coverslips (washed once in Medium-199, Hyclone/thermo Fisher, Waltham, MA) at a shear rate of 300 s⁻¹ as described.⁴ Formation of VWF-platelet strings was visualized using an Axio Observer.Z1 microscope with Axio Vision release 4.6.3 software (Zeiss). After 2.5 minutes of perfusion, 6 snapshots were taken within a 30-s period. Number of strings/field and number of platelets/micron were determined via manual inspection of images.

**In vivo thrombosis and string formation:** In vivo analysis of VWF-platelet string formation and ferric-chloride induced thrombus formation was performed on mesenteric vessels that were prepared as described¹⁻⁵, except that Rhodamine-6G was used to label platelets fluorescently. VWF-platelet-string formation was induced by the topological application of A23187 (10 µM) to the mesenteric venules, and platelet adhesion to the endothelial surface was recorded for 10 min. VWF-platelet strings were defined as the linear alignment of at least three platelets for a period of at least 2 seconds. Ferric-chloride injury of mesenteric arterioles was induced via the application of a filter soaked in 7.5% FeCl₃. Platelet accumulation was recorded for 45 min from the time of filter placement. Time to initial thrombus formation with a diameter ≥30 µm and time to occlusion were determined. Recordings were made using a 10x/0.3NA air-objective.
Statistical analysis: Data are expressed as mean ± SD, unless stated otherwise. Statistical analysis was performed with the Student unpaired t-test, and Welch correction was applied when appropriate. Multiple comparisons were performed using one-way ANOVA with Dunnett’s multiple comparison test. P-values less than 0.05 were considered statistically significant.

References supplementary material:


**Supplementary figure I:**

Resting HUVECs were incubated with a mixture of murine monoclonal antibodies against VWF in combination with polyclonal goat antibodies against histone-H3 (panel A) or osteoprotegerin (OPG; panel B). Bound antibodies were simultaneously detected by the application of oligonucleotide-coupled secondary antibodies, the sequence of which was complementary. Following amplification, amplified oligonucleotides were highlighted using fluorescent-labeled probes, generating discrete red fluorescent spots for each protein complex. Fluorescence intensity was quantified using ImageJ-1.44 software package (http://rsbweb.nih.gov/ij/index.html) and is shown in panel C. Data represent the mean fluorescent intensity ± SEM of 12-16 cells, obtained from two independent experiments.