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Structure-function relationships of von Willebrand factor

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Nomenclature

DAB	diamino benzidine
GP Ib	glycoprotein Ib (CD42b)
GP IIb/IIIa	glycoprotein IIb/IIIa (CD41/CD61, $\alpha_{IIb}\beta_3$)
GP VI	glycoprotein VI
HRP	horseradish peroxidase
moAb	monoclonal antibody
PVDF	polyvinylidene fluoride
RD	reflective density
RF	relative mobility
RIPA	ristocetin induced platelet agglutination
scFv	single-chain variable fragment
Tris	tris(hydroxymethyl)aminomethane, also known as THAM
V_H	heavy chain variable region
V_L	light chain variable region
VWF	von Willebrand Factor

Introduction

Blood provides the effective means of transportation between the organs. To fullfill this task, it is distributed to the tissues in a network of blood vessels, which is closed in vertebrate. Failure to meet this requirement is incompatible with sustaining life functions, therefore it is essential to have effective counter-measures if leakage would occur. The need for intervention is obvious when blood vessels are damaged, but also the same mechanisms are utilised when the endothelial lining of blood vessels is renewed periodically. While it is important to seal the sites of injuries, it is also essential to maintain the blood in a fluid state elsewhere. The first goal is achieved by a sequence of interdependent events, involving both cellular and extracellular elements. Immediately after the injury the contraction of the vessel and the adhesion of platelets to the subendothelial structures temporary closes the wound, in the same time the coagulation cascade launches and a haemostatic plug is formed which is later degraded while the vessel wall is restored. The second goal requires these interwoven processes to be coordinated and controlled spatially and temporally; most importantly only the appropriate triggers should be able to activate this system. The interest of the tissues served by the damaged vessel also requires the size and the lifetime of the haemostatic plug to be kept within reasonable limits. The complex system realising all these functions is called haemostasis

The adhesion of platelets to the damaged vessel wall is one of the earliest steps of haemostasis. As soon as the endothelium is compromised, platelets are recruited on the exposed subendothelial structures. In veins or larger arteries where the shear forces are lower, platelets bind to the subendothelial collagen directly via their GP VI and GP IIb/IIIa receptors. However, when platelets are exposed to larger wall shear stress, especially in arterioles and stenosed vessels, these receptors are unable to support adhesion alone. This observation suggests, that under these conditions there is an additional mechanism that slows down the moving platelets

and withstands the shear forces so platelets are able to form stable bonds with the disrupted vascular surface. This process was visualised in real time microscopic systems where it has been demonstarted that platelets roll on the exposed subendothelium before they come to a stop, and that this phenomenon depends on the interaction of von Willebrand factor (VWF) and its platelet receptor glycoprotein GP Ib. The significance of this interaction is confirmed by the fact that the characteristic bleeding symptoms (e.g. mucosal bleeding) of VWD type 3 patients (lacking VWF) and patients with Bernard-Soulier syndrome (lacking GP Ib) appear in vessels where the wall shear stress is high.

VWF acts as a bridge between the subendothelial collagen and the platelets. The VWF-GP Ib interaction has some notable properties making it particularly fit for its purpose. VWF is built up from multiple subunits each one with a binding site for GP Ib and collagen, this way it provides a high local density of aligned binding places. Also the GP Ib-VWF bond has a fast on and a fast off rate and a biphasic response to forces acting upon it, that allows the platelets to roll, adhere or detach in response to shear forces. Interestingly, unlike the selectins and their receptors, which mediate leukocyte rolling, both VWF and its receptor is present in the bloodstream in the same time. However, under normal circumstances native GP Ib has an extremely low affinity towards VWF, they only interact if pathologically high shear forces are present or VWF is immobilised.

This observation implies the existence of a regulatory mechanism. Present theories about the regulation of this interaction hypothesise conformational changes at different scales of VWF (affecting the affinity or accessibility of the binding site) and/or emphasise the control of multimer size (altering avidity):

- Shear stress may induce conformational changes near or in the GP Ibα binding site that enhances its affinity towards GP Ibα. Almost all reported gain of function mutations are missense mutations located to the A1 domain, which contains the only known GP Ibα binding site in VWF. These mutations are thought to act through the destabilisation of the base of the A1 domain and thereby mimicking the structural

changes caused by shear stress. It has been also demonstrated using molecule modeling that the mechanical stress changes the orientation of GP Ib and the A1 domain in a fashion that a new salt bridge forms resulting in a stronger interaction.

- Shear forces also may cause larger scale conformation changes, not directly involving the conformation of the GP Ibα binding site. The isolated A1 domain spontaneously binds to GP Ibα suggesting that other domains also modulate the VWF GP Ib interaction. In line with this observation the antibody 1C1E7 is reported to bind to the N-terminal region of VWF (D'D3 region), but it induces VWD type 2 like alterations. Small angle neutron scattering studies also suggest local rearrangements at the domain level in response to fluid shear.

- VWF in solution has a coiled structure causing a large fraction of the GP Ib binding sites to be buried. It is proposed that shear forces and/or immobilisation uncoils VWF, making the binding sites accessible. Some studies suggest, that the the drag forces exerted by platelets in flow are also needed for uncoiling.

- The size of the VWF multimers also affects the VWF GP Ib interaction. It is generally accepted that larger multimers are more effective at supporting platelet adhesion which is ascribed to the increased avidity due to the higher number of binding sites. It is not known if and how the multimer size distribution is regulated during synthesis. However, under shear conditions ADAMTS13 can specifically cleave secreted (UL)VWF. This suggests that ADAMTS13 limits platelet adhesion by decreasing multimer size at sites of high shear flow.

This field is extensively researched, as deficient regulation can be associated with bleeding disorders and thrombotic events as well, furthermore it is a potential target of antiplatelet/antithrombotic drugs aiming on the high shear flow areas (such as stenosed arteries and stents). A definitive answer has not yet been found, perhaps a combination of several present theories will explain the gathered experimental data.

Objectives

Our aim is to study the importance of interdomain interactions in the modulation of the affinity of VWF towards GP Ib, and to develop novel methods enabling further studies in this field.

- Analyse the role of the D'D3 region in regulating the VWF – GP Ib interaction:
  - Explore the mechanism of action of the monoclonal antibody (moAb) 1C1E7. This antibody has its binding site in the D'D3 region — outside of the GP Ib binding domain (A1) — but still it enhances the VWF GP Ib interaction
  - Cross-blocking studies using monoclonal antibodies binding to different domains of VWF
  - Studies with recombinant deletion mutants of VWF
- Development of novel methods:
  - Improve and extend the methods of VWF multimer distribution analysis, including electrophoresis and data analysis techniques
  - Establish a system, where platelets are substituted with similar sized corpuscules bearing functionally active GP Ibα. This would enable the study of the effect of platelets dragging VWF under flow conditions on the conformation of VWF.

Materials and Methods

Interdomain interactions

Construction and expression of 1C1E7 single-chain variable fragment

Total cell RNA was extracted from 1C1E7-expressing hybridoma cells and was reverse-transcribed using oligo$(dT)_{20}$ primers. Degenerate sense primers (based on the amino acid sequences of N-terminal sequence of the heavy and light chain of 1C1E7) and framework-specific antisense primers were used for the amplification of for the heavy and the light chain variable regions (V_H and V_L, respectively). The single-chain variable fragment (scFv) coding sequence (V_H–$(G_4S)_3$–V_L) was constructed by splice overlapping extension PCR after extendending both V_H and V_L appropriately. The 1C1E7 scFv was expressed in *Escherichia coli* as a His-tag fusion protein.

Platelet agglutination

For the agglutination experiments using 1C1E7 scFv, citrated blood was used to obtain platelet-rich plasma. Platelet-rich plasma was incubated for 3 min with buffer and with 1C1E7 scFv or IgG, after which agglutination was induced by the addition of ristocetin. For the agglutination experiments using the VWF fragments, washed platelets were used. ΔD'D3 (lacking D'D3 region) ± or D'D3, plusD'D3 (control containing D'D3) were added to a final concentration of 10 μg/ml, and the mixture was incubated for 3 min, after which agglutination was induced by the addition of ristocetin.

Cross-blocking analysis

Monoclonal antibodies 1C1E7, 418 (binding site in D'D3), 701, 724 (binding site in A1) and 82D6A3 (binding site in A3) were used for these experiments.

"Immobilised" VWF: Microtiter plates were coated with VWF and one biotinylated and one competing (non-biotynilated) moAb was incubated in the wells. The amount of biotinylated moAb was detected with peroxidase-labelled streptavidin

"Soluble" VWF: Microtiter plates were coated with one of the moAbs. Biotinylated VWF was preincubated with one of the moAbs after which this solution was transferred to the coated wells. After a further incubation of 30 min at 37 °C, the amount bound b-VWF was detected as above.

VWF multimer distribution

SDS agarose gelelectrophoresis and immunoblotting

VWF molecules were separated either by using the modified Laemmli buffers or by a Tris-Borate discontinuous buffer system. Low resolution (0.8%) agarose running gels (12.5 cm×10 cm×1.2 mm) were cast on glass plates, the first 2.5 cm strip was replaced with the stacking gel. After denaturation, electrophoresis was performed at constant current for either 6 hours at 20 mA/gel, or overnight at 6 mA/gel on a plate cooled to 18°C. The proteins were transferred to PVDF membranes using the tank electro-blotting method. To enhance the transfer, VWF multimers were partially degraded by 1 mM β-mercaptoethanol (mercaptolysis) before blotting as reported by Bowen and Bowley. VWF was visualised by immunostaining using HRP labelled polyclonal rabbit anti-human VWF antibody and DAB substrate.

Quantitative multimer analysis

Digital images of the membranes were obtained by a calibrated densitometer, the resulting reflective density (RD) against relative mobility (RF) data

was processed by a software developed by us. First the RD versus RF curves were smoothed and VWF peaks were identified. The quantitative analysis was performed using two methods.

With the first method ($\mathbf{M_{10}}$) the relative amount of large multimers — defined as oligomers larger than the icosamer (band 10) — was calculated.

With the second method ($\mathbf{M_{MW}}$) the degree of multimerization was characterised. It was assessed by the molecular weight corresponding to the lower boundary of the largest 25% of VWF protein. The molecular weight corresponding to this RF was estimated based on the correlation of the VWF peaks mobility and their molecular weight. The 25% boundary was chosen after comparing the M_{MW} of groups of samples lacking large multimers, normal samples and samples containing unusually large multimers using different boundaries.

The processing of densitometric data did not involve manual steps in either of the calculations.

GP Ibα microspheres

24B3 is a moAb capable of capturing glycocalicin from plasma in a functionally active comformation. (glycocalicin is a highly glycosylated, hydrophilic135 kDa proteolytic fragment of GP Ibα). 24B3 was covalently immobilised onto 3 μm carboxylate polysterene microspheres according to the manufacturer's protocols. As glycocalicin source outdated human platelets were used, 24B3 coated beads were incubated with the filtrate of washed and sonicated platelets overnight. The number of 24B3 coupled to the microspheres and the number of bound glycocalicin molecules was counted. Purified VWF and Botrocetin was added to the beads, and incubated for 2 h. Samples were fixed with 1% paraformaldehyde. Finally, the sample was incubated with rabbit anti-VWF polyclonal antibody, and subsequently with swine anti rabbit IgG-FITC. Bound VWF was detected using flowcytometry.

Results

Interdomain interactions

Platelet agglutination studies

Previous studies has suggested, that the GP Ibα VWF-A1 interaction might also be modulated by other domains. The moAb 1C1E7 binds to the D'D3 region of VWF and it can still positively modulate the binding of GP Ibα to VWF. This suggests a functional role of D'D3, which we studied using the following dimeric recombinant VWF constructs: ΔD'D3 (lacks the D'D3 region but still contains the N-terminal flanking region of the VWF A1 domain), plusD'D3 (lacking D4-CK region, used as control possesing the D'D3 region and the A domains) and D'D3 (containing only the D'D3 domains). At lower platelet concentrations, ΔD'D3 was able to sustain spontaneous platelet agglutination in contrast to plusDD3. The threshold dose needed for ristocetin induced platelet agglutination (RIPA) of washed platelets was lower for ΔD'D3 than for plusD'D3, demonstrating the inhibitory effect of the D'D3 region on the interaction of VWF with GP Ibα. In line with this, D'D3 inhibited RIPA supported by ΔD'D3 while having no effect on plusD'D3. These results show that the deletion of the D'D3 region ΔD'D3 could be reverted by external addition of these isolated D'D3 domains.

Cross blocking studies

We have verified the structural proximity of the D'D3 region with the A1 domain, by testing whether the binding of moAb 418 (interacting with the D'D3 region) could be blocked by moAbs 701 and 724 (both interacting with the A1 domain) both using VWF in solution and immobilised VWF. As a negative control, moAb 82D6A3 (interacting with the A3 domain)

was used. Two different enzyme-linked immunosorbent assay like set-ups were developed, (i) cross-blocking of binding of b-418 to immobilised VWF by unlabelled moAbs and (ii) the inhibition of the capture of b-VWF to immobilised moAb 418 by the unlabelled moAbs. When VWF was immobilised, no measurable inhibition of the binding of b-418 was observed by any of the moAbs except with unlabelled 418 as the positive control, although all moAbs were able to interact with immobilised VWF. In contrast, moAbs 701 and 724 did compete with moAb 418 for the binding to soluble b-VWF, whereas moAb 82D6A3 had again no effect.

Construction and Expression of 1C1E7 scFv

1C1E7 scFv was expressed as a His-tagged fusion protein in E. coli. 1C1E7 scFv increased ristocetin-induced platelet aggregation, similarly to 1C1E7 IgG, providing evidence that the construction was correct. Comparison of the primary sequence of the complementarity determining region 3 of the 1C1E7 heavy chain revealed a strong similarity with the N-terminal flanking region of the VWF A1 domain.

VWF multimer distribution

Comparison of buffers

We detected 18 to 22 discrete bands in normal samples when using the Tris-borate buffers. Fewer bands were resolved with the Laemmli buffers, where also a distorted band near the running front was commonly visible. To compare the resolving power of the gels, and to elucidate the contents of the distorted band, a sample was separated in a two-dimensional electrophoresis with a Laemmli gel in the first dimension and a Tris-Borate gel in the second. The separation of large multimers was superior in the direction of Tris-Borate buffers, and also the distorted band in the Laemmli gel resolved into multiple bands.

Optimisation of M_{MW} calculation

After optimisation, M_{MW} was defined as the molecular weight of the lower boundary of the largest 25% of VWF protein (M_{MW}). The 25% limit was established by evaluating a subset of data (7 platelet lysate, 11 normal and 8 VWD 2B samples) using a range of limits between 1-80%. The method's ability to distinguish between the groups of samples (platelet lysate, normal and VWD type 2) was evaluated by the t-statistic. At smaller thresholds the discrimination between the platelet lysate and normal groups increased, whereas the best discrimination between the normal and VWD 2B groups was observed when calculating with larger thresholds. The 25% threshold is equally spaced from the threshold where platelet lysate (10%) and where VWD type 2 samples (40%) are separated better from normal samples. At this threshold the statistical difference between groups is highly significant (mean±SD 10.42±0.78, 6.27±0.68, 2.13±1.12 MDa for platelet lysate, normal and VWD type 2 group respectively, $p<<0.01$)

Method Characteristics

Reproducibility, expressed as the coefficient of variation of M_{MW} and M_{10} measurements of the same normal control sample on different days (n=20) was 7.8% and 15.9% respectively. The M_{MW} and M_{10} of 35 healthy individuals was 6.1±0.72 and 33.5±7.9 (mean±SD). The methods' tolerance towards the variations in the intensity of immunostaining was studied by evaluating a series of dilutions of one sample between $0.25-2.5$ times the standard dilution. The different dilutions yielded an average RD between 0.061 and 0.319 for the VWF containing part of the lanes, M_{MW} or M_{10} results were not affected in this range.

Correlation of M_{MW} and M_{10}

We evaluated M_{MW} and M_{10} of platelet lysate, control samples, VWF degraded by ADAMTS13 and samples from VWD type 2 patients. The VWF in platelet lysate is protected from plasma proteases and consequently it

contains larger multimers, whereas VWD type 2 patients typically have less or no large multimers. We found good correlation between the degree of multimerization and the amount of large multimers in normal samples (r^2=0.98) and platelet lysate, however the slope of the regression lines were different ($p<0.001$). Characteristically VWF in platelet lysate contained larger multimers than healthy samples with the same amount of large multimers.

Correlation of M_{MW} with Collagen binding activity and Ristocetin cofactor assay

Results of the functional tests of VWF — collagen binding activity and ristocetin cofactor assay — were compared to M_{MW} on normal and VWD type 2 samples. The functional assays to antigen ratios were at least partially determined by multimerization (r^2=0.42 and 0.43 for VWF:RCo and VWF:CB respectively). Four patient samples (16%) had normal M_{MW} but abnormal functional assays, these cases are possibly VWD type 2M. On the other hand three (12%) VWD type 2 patients had normal VWF:RCo to antigen and five (20%) type 2 patients had normal VWF:CB to antigen ratios but abnormal M_{MW}. Some of these samples (1 in case of VWF:RCo and 3 in case of VWF:CB) had low antigen levels, where the reproducibility of the functional assays tends to diminish. Furthermore five (19%) of normal samples had abnormal VWF:RCo to VWF:Ag ratio, while none of these samples had abnormal M_{MW}or VWF:CB to VWF:Ag values.

GP Ibα microspheres

Polystyrene beads coated with 24B3 have acquired the ability to bind glycocalicin and monoclonal anti-GP Ibα antibodies. We have found that the number of coupled 24B3 (~64000) was only partially saturated with glycocalicin (number of bound glycocalicin molecules ~3140). The significantly lower number of detectable glycocalicin molecules indicates that not all of the immobilised 24B3 is in a binding conformation. VWF was incubated

with the 24B3 beads preincubated with glycocalicin and VWF was detected using a two-step labelling method. VWF signal was detectable on the beads, which demonstrated that the glycocalicin captured by 24B3 is capable of binding to VWF. The specificity of the measurement was confirmed by omitting glycocalicin, which resulted in loss of VWF signal.

Discussion

The primary role of platelets can be summarised simply: "Platelets plug holes in blood vessels". More specifically, in the first step of this process platelets in the arterial side of circulation identify damaged vascular lining and stick to the areas of exposed subendothelium. Platelets in the capillaries move with an average velocity of 0.5 mm/s, this together with the fact that their diameter is approximately 2 μm means that they spend only approximately 4 ms above a given point of the vessel wall. This is a roughly the timeframe in which platelets have to identify injured vessel segments and adhere to it. The solution is that platelets at high shear rates have a translocation phase between initial contact and firm adhesion. In this process VWF acts as a bridge between the fast moving platelets and the stationary vessel wall and enables their interaction. Interestingly VWF is present in the bloodstream together with platelets, but their interaction is only observed under very high shear conditions normally not present. *In vitro*, binding of VWF to GP Ib can be induced by modulators such as ristocetin or botrocetin, although under these conditions binding is induced rather artificially. Even so it has been demonstrated that ristocetin dependent interactions quite closely correlate with the physiological shear-dependent situation.

However *in vivo* binding is induced by the immobilisation of VWF and by exposure to shear. These observations suggest that the affinity toward GP Ib is regulated by conformational changes in VWF that are induced by shear and immobilisation and lead to exposure of functional sites. We have studied the possible role of interdomain interactions in this process by exploring the mechanism of action of the moAb 1C1E7. This moAb interacts with the the N-terminal D'D3 domains in VWF but it is capable of increasing the affinity of VWF towards GP Ib, although its binding site is distant from the GP Ib binding site. This would suggest a modulator effect for the binding region of 1C1E7 in VWF on the GP Ib interaction, therefore

we aimed to determine the functional role of the D'D3 region in binding of VWF to GP Ib. Deletion of the D'D3 region resulted in a higher affinity for GP Ib as demonstrated by the lower ristocetin threshold of the dimeric constructs ΔD'D3 (lacking the D'D3 region) compared to plusD'D3 (lacking the C-terminal region, but having intact D'D3 domains) in RIPA studies. Moreover, at lower platelet concentrations, ΔD'D3 was able to sustain spontaneous platelet agglutination in contrast to plusDD3. All together, these data strongly suggest that the D'D3 region may act as an inhibitory region, shielding the A1 domain in VWF. Our data further confirm and extend previous studies that demonstrated the putative shielding of the GP Ib binding site in the A1 domain by the N-terminal flanking regions of the A1 domain and the C-terminal region of the D3 region. To further substantiate the idea that DD3 would interact with A1 in VWF in solution and no longer when immobilised / sheared, we looked to whether moAbs against the respective domains would block each others binding or not when VWF is in solution or immobilised. The anti-DD3 moAb 418 cross-competed with the anti-A1 domain moAbs 701 and 724 when VWF was in solution, but not on immobilised VWF, providing evidence for a changing distance between the domains upon immobilisation. Finally, we also found a striking sequence similarity between the primary sequence of the complementarity determining region 3 of the 1C1E7 heavy chain and the N-terminal flanking region of the VWF A1 domain. This N-terminal flanking region is shown to be important in modulating the binding of the A1 domain with GP Ib as modifications or deletions in this region increase the affinity of VWF for GP Ib. In our view this might be the region within the A1 domain that interacts with the D'D3 region, and it is possible that 1C1E7 competes with the N-terminal flanking region for this site and disrupts their interaction. These results demonstrate an inhibitory role for the N-terminal region in VWF for the GP Ib interaction. Based on these observations, the following hypothesis might be put forward. In native, resting conditions, the A1 domain and the D'D3 region are in close proximity, possibly through an interaction of the N-terminal flanking region of the A1 domain with the D3-domain. This interaction is limiting the accessibility of the GP Ib binding

site. When VWF is immobilised, this interaction is disrupted through conformational changes in VWF — possibly in the DD3 — region which allows recruitment of platelets through their GP Ib receptors.

VWF in solution has a coiled structure causing a large fraction of the GP Ib binding sites to be buried, this effect may largely be dependent on VWF multimer size. Large multimers have higher avidity because of the larger number of binding sites as well. In line with these observations there is a strong association between the presence of large multimers and the haemostatic function in case of VWF. The amount and presumably the multimerization of VWF oligomers released by the endothelial cells and the activity of the plasma enzyme ADAMTS-13 degrading the oligomers have a high inter-individual variability and known to change in pathological conditions. Consequently, the size and the amount of the large oligomers, which are main determinants of the haemostatic activity of VWF, are expected to differ as well. The established method to quantify the amount of the large oligomers is to calculate the percentage of VWF protein larger than the icosamer (10th band), though other approaches also exist. However, the identification of VWF bands is not reliable with the commonly used Laemmli buffers as the separation of the small VWF oligomers is inadequate. The transfer of large multimers is also inconsistent with the traditional blotting techniques. After evaluating several types of buffer systems we found that Tris-borate buffers combined with mercaptolysis-aided blotting is better suited for this purpose. The characterisation of the degree of multimerization poses some problems. The size of the largest oligomer in the sample could be used as an obvious measure, however its quantity is so low that is not discernible from the background above VWF bands. Furthermore the size of this oligomer is in the range of 20 MDa, which is not resolved by agarose gels, so even if it was detected, the determination of its molecular weight would not be possible. These problems can be solved by detecting a proportion of the largest oligomers instead of just detecting the largest. On the other hand too large proportions may decrease selectivity. By comparing normal samples to either having less (VWD type 2) or having extra large (platelet lysate) multimers, we have found that at 25% the

method is selective and the interference by the background is acceptable (the latter problem affects VWD type 2 samples more, as the VWF bands are further from the sample application wells). Further study of platelet lysate confirmed that the larger oligomers are detectable, even if the amount of large oligomers is the same as in plasma samples. To further evaluate M_{MW}, we investigated the relationship between M_{MW} and GP Ib binding (VWF:RCo) and collagen binding (VWF:CB) functions in healthy and VWD type 2 samples. There was moderately strong (r^2~0.4) relationship between M_{MW} and both of the functional assays, the higher sensitivity of collagen binding to the lack of large multimers was not evident. It is worth noting, that the high variability of the functional test at low antigen concentrations may result in normal activity-to-antigen ratios in cases with abnormal electrophoresis results. These results demonstrate, that our new electrophoresis and analysis techniques are accurate and reproducible allowing further studies of the importance of VWF multimer distribution.

It is proposed that shear forces and/or immobilisation uncoils VWF, making the binding sites accessible. However not all studies were able to confirm this hypothesis, suggesting that the binding of platelets to VWF, and the drag forces exerted on VWF by them in flow is also needed for uncoiling. Platelets under shear conditions become activated when binding to VWF or to the subendothelial structures. This renders the exploration of the above mentioned effect more difficult. We have planned to substitute platelets with similarly sized corpuscules, bearing functionally active GP Ibα to circumvent this problem. We have covalently coupled moAb 24B3 to 3 μm carboxylate polysterene beads, which is known to bind recombinant GP Ibα and glycocalicin (cleaved off extracellular part of GP Ib in plasma) in an active conformation. We have demonstrated that these beads were capable of capturing glycocalicin from platelet lysate, and were capable of binding VWF if botrocetin was added. This confirms that these beads carry functionally active GP Ibα, and may turn into a valuable tool for further flow studies.

Summary of new findings

- We have shown that the D'D3 domain inhibits the interaction of GPIbα and VWF-A1:
  - Cross-blocking studies demonstrated the proximity of D'D3 and A1 in solution, but not when immobilised
  - Ristocetin induced platelet agglutination studies demonstrated the inhibitory role of this region
  - The similarity of the peptide sequence of 1C1E7 and the A1 domain suggests, that they have a common bind site in D'D3. This would explain how 1C1E7 enhances the affinity of A1: 1C1E7 acts by disrupting the shielding of the A1 domain by D'D3.
- We have developed new electrophoresis and analysis techniques to quantitatively analyse the multimer distribution of VWF. We have demonstrated that these methods are accurate and reproducible:
  - We have assessed the sensitivity of this method to the presence of ultra large multimers and its utility on samples lacking large multimers.
  - We have compared the degree of multimerisation and the results of functional test in case of normal and VWD type 2 samples.
- We have developed a system to model the drag forces exerted by platelets on VWF. We have coated polysterene beads with glycocalicin, and we have demonstrated, that these beads are capable of binding VWF.

Original Publications

Publications used in preparation of the thesis

1. **Udvardy, M. L.**, Szekeres-Csiki, K., and Hársfalvi, J. (2009) Novel evaluation method for densitometric curves of von Willebrand Factor multimers and a new parameter (M_{MW}) to describe the degree of multimerisation. *Thromb Haemost 102*, 412–417.
   Impact factor: 3.803 (2008)

2. Ulrichts, H.*, **Udvardy, M. L.***, Lenting, P. J., Pareyn, I., Vandeputte, N., Vanhoorelbeke, K., and Deckmyn, H. (2006) Shielding of the A1 domain by the D'D3 domains of von Willebrand factor modulates its interaction with platelet glycoprotein Ib-IX-V. *J Biol Chem 281*, 4699–4707.
   *Both authors contributed equally to this work
   Impact factor: 5.808

Total impact factors of publications used in preparation of the thesis: 9.611

Publications related to the thesis

1. Szarvas, M., Oparaugo, P., **Udvardy, M. L.**, Toth J., Szanto T., Daroczi L., Vereb G., and Harsfalvi J. (2006) Differential platelet deposition onto collagen in cone-and-plate and parallel plate flow chambers. *Platelets 17*, 185-90.
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Impact factor: 2.271 (2008)

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Impact factor: 3.803 (2008)

Total impact factors of publications related to the thesis: 7.753

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