Plasma glycocalicin as a source of GPIbα in the von Willebrand factor ristocetin cofactor ELISA

Karen Vanhoorelbeke, Inge Pareyn, Agota Schlammadinger[1], Stephan Vauterin, Marc F. Hoylaerts[2], Jef Arnout[2], Hans Deckmyn

Laboratory for Thrombosis Research, IRC, KU Leuven Campus Kortrijk, Kortrijk, Belgium [1]2nd Department of Medicine, University of Debrecen, Debrecen, Hungary

[2]Center for Molecular and Vascular Biology, KU Leuven, Leuven, Belgium

Summary

We have previously demonstrated that the von Willebrand factor ristocetin cofactor activity (VWF:RCo), used in the diagnosis of von Willebrand disease (VWD), can be accurately determined via ELISA by measuring the ristocetin-induced binding of VWF to a captured recombinant fragment of GPIbα (rfGPIbα,AA 1–289) (Vanhoorelbeke et al., *Thromb Haemost* 2000; 83: 107-13). This ELISA is more reliable than the currently used platelet agglutination test. Normal plasma contains relatively high concentrations of glycocalicin, a proteolytic fragment of GPIbα. We therefore studied whether non-purified plasma glycocalicin can replace rfGPIbα in our ELISA. Of 42 anti-GPIbα monoclonal antibodies (MAbs) capable of binding plasma glycocalicin, only one MAb captured glycocalicin in a spatial orientation exposing the VWF-binding site in glycocalicin, allowing a specific and dose-dependent ristocetin-mediated VWF-binding. Intra- and inter-assay variability were comparable with those for the rfGPIbα based VWF:RCo ELISA. The VWF:RCo activity of plasma from 33 normal individuals, 19 type 1, 16 type 2A, 9 type 2B, 8 type 2M and 7 type 3 VWD patients was determined with this ELISA and allowed a clear identification of VWD patients. Furthermore, determination of the VWF:RCo/VWF:Ag ratio resulted in the discrimination between type 1 and type 2 VWD patients. Results for the glycocalicin based and the rfGPIbα based VWF:RCo ELISAs were in good agreement (r = 0.943). There was also a good correlation between the glycocalicin based ELISA and the standard platelet agglutination test (r = 0.963). In conclusion, to diagnose VWD, a VWF:RCo ELISA based on antibody immobilized plasma glycocalicin can be performed reliably.

Keywords

Plasma glycocalicin, von Willebrand disease (VWD), von Willebrand ristocetin cofactor activity (VWF:RCo) assay

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Introduction

Von Willebrand disease (VWD) is one of the most frequent inherited bleeding disorders characterized by typical mucocutaneous bleeding symptoms (epistaxis, menorrhagia). VWD is due to quantitative (type 1 and type 3) and/or qualitative (type 2A, 2B, 2M and 2N) deficiencies (1) in the plasma glycoprotein von Willebrand factor (VWF), a protein known to play a role in primary haemostasis (2, 3). VWF is a multimeric protein with a M_r of up to 20 million and is synthesized by endothelial cells and megakaryocytes. By forming a bridge between the platelets through glycoprotein (GP)Ib/V/IX and the exposed collagen in the damaged vessel wall, VWF is responsible for the initiation of platelet plug formation especially in regions of high shear stress. VWF also binds coagulation factor VIII (FVIII) thus protecting it from degradation.

Different laboratory tests aim at correctly diagnosing VWD, such as the bleeding time (BT), the VWF antigen level (VWF:Ag) determination, the VWF ristocetin cofactor activity (VWF:RCo) measurement and the VWF collagen-binding activity (VWF:CBA) measurement. For the identification of the different subtypes of VWD additional tests such as multimer analysis, ristocetin-induced platelet agglutination and FVIII-binding assays are needed (3).

The VWF ristocetin cofactor assay (VWF:RCo) measures the activity of VWF in plasma. In the presence of ristocetin (4, 5), high VWF multimers then acquire a higher affinity for platelet GPIb/V/IX (6). The standard VWF:RCo techniques measure the

Correspondence to:
Karen Vanhoorelbeke
Laboratory for Thrombosis Research
IRC, KU Leuven Campus Kortrijk
E. Sabbelaan 53, 8500 Kortrijk
Belgium
Tel.: + 32 56 246422, Fax: + 32 56 246997
E-mail: Karen.Vanhoorelbeke@kulak.ac.be

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agglutination of fixed donor platelets with patient plasma in the presence of ristocetin (7, 8). However, this assay suffers from high inter-assay and intra-assay variability due to the use of donor platelets and the sometimes difficult interpretation of the platelet agglutination profile (9). To improve the diagnosis of VWD, we have recently proposed a new and more reproducible ELISA to determine the VWF:RCo activity (10, 11). In this assay, a recombinant fragment of GPIbα (rfGPIbα AA 1–289), that contains the VWF binding site of the receptor, is captured on an anti-GPIbα monoclonal antibody (MAb). Different dilutions of patient plasma are added in the presence of a fixed concentration of ristocetin and bound VWF is detected using polyclonal anti-VWF antibodies labelled with horse radish peroxidase (HRP). The VWF:RCo in the patient plasma is calculated by comparison to VWF-binding in a normal plasma pool.

We have now extended the applicability of this ELISA by demonstrating that plasma glycocalicin can be used instead of rfGPIbα to mediate the ristocetin-induced recruitment of VWF to GPIbα. Glycocalicin is a hydrophilic proteolytic fragment of GPIbα with a M_r of ~ 135 000 known to be highly glycosylated (56.5% (w/w) carbohydrate) (12). The platelet derived calcium dependent protease calpain was believed to be responsible for the proteolysis of GPIbα (13, 14), but recently a role for matrix metalloproteases (MMP) has been demonstrated (15, 16). Glycocalicin is found in normal plasma at a concentration of 1–3 μg/ml (17, 18) and plasma levels may reflect the turnover of platelets and would therefore be an aid in the classification of thrombocytopenic disorders (19, 20). Glycocalicin used in the present study was not purified from plasma but captured on an anti-GPIbα MAb. The choice of such antibody however was critical as we found only one single MAb capable of presenting glycocalicin in an optimal conformation necessary to allow ristocetin-induced VWF-binding.

Materials and methods

Materials

The plasma pool of 25 normal plasmas as a source of VWF was prepared as described and was therefore considered to contain 10 μg/ml VWF and 1 U/ml VWF:RCo (10). Citrated Platelet Poor Plasma (PPP) used as a source of glycocalicin was obtained from single normal individuals and was prepared as described (10). Plasmas from VWD patients were either from the 2nd Department of Medicine, University of Debrecen, Hungary or were a kind gift of Dr. K. Peerlinck (Center for Molecular and Vascular Biology, KU Leuven, Leuven, Belgium). Plasmas were always incubated for 5 min at 37°C before use. Purified VWF was from the Red Cross Belgium. The rfGPIbα was obtained from CHO cells expressing soluble rfGPIbα and was purified and characterized as described (10). Ristocetin was from American BioProducts (abp) (New Jersey, USA).

Monoclonal antibodies (MAb) and platelet glycocalicin

Monoclonal anti-GPIbα antibodies were prepared in house by immunizing Balb/C mice with purified GPIbα (21). Anti-GPIbα MAb 2D4 captures rfGPIbα in an active conformation (10), MAb 6B4 inhibits the ristocetin-induced VWF-binding to rfGPIbα (AA1–298) (22) and MAb 11A11 is a neutral anti-GPIbα antibody. MAbs were biotinylated using NHS LC biotin (sulfosuccinimidyl 6-[biotinamido] hexanoate) Pierce (Rockford, IL) according to the manufacturer's instructions.

Platelet glycocalicin was isolated from 20 units outdated platelets (Red Cross, Roeselare, Belgium) as described using 3M KCl (23) and purified on a Wheat germ Lectin Sepharose® 6MB column (Amersham Biosciences, Uppsala, Sweden) followed by loading on a Resource Q column (Amersham Biosciences).

VWF:RCo ELISA using plasma glycocalicin

Microtiter plates (96 well, Greiner, Frickenhausen, Germany) were coated overnight with anti-GPIbα MAbs (5 μg/ml in phosphate buffered saline (PBS)) at 4°C. Wells were blocked with 3% milk powder in PBS for 2 hours at room temperature (RT). Anti-GPIbα MAbs were saturated with a constant amount of glycocalicin using a ½ dilution of PPP (in TBST) for 1.5 hour at 37°C. A ½ dilution series of plasma pool (as a VWF source and consisting of a pool of 25 normal plasmas, cfr. above) starting from a 1/32 dilution (in TBST) was added in the presence of a constant amount of ristocetin for 1.5 hour at 37°C. Bound VWF was detected using rabbit anti-human VWF antibodies labelled with HRP (anti-VWF-Ig-HRP) (Dako) for 1 hour at RT. Visualisation was performed with orthophenylenediamine (OPD, Sigma, St. Louis, USA) and H_2O_2. The colouring reaction was stopped with 4M H_2SO_4. After each incubation step, plates were washed 3 times with TBST after coating and blocking steps and 9 times elsewhere.

To determine the VWF:RCo activity in normal plasmas or plasmas of VWD patients, a standard curve was constructed from the mean data of 4 assays where each time 6 dilutions (1/32, 1/64, 1/128, 1/256, 1/512, 1/1024) of the normal plasma pool were tested. In the normal plasma pool, the VWF:RCo was set as 1 U/ml. The linear part of the curve was fitted using linear regression and the equation was used to calculate the VWF:RCo in normal or patient plasmas. To obtain these values, 4 dilutions (1/32, 1/64, 1/128 and 1/256) of normal or patient plasma were tested in duplicate in the same ELISA assay as the plasma pool and the VWF:RCo values of the 1/128 and 1/256 dilutions (dilutions situated in the linear part of the standard curve) were calculated using the standard curve. The mean of these 4 data resulted in the VWF:RCo in these plasmas. Four dilutions of patient plasma are performed in order to have a good estimate of the quality of the binding curve.

The VWF:RCo ELISA using rfGPIbα was performed and calculated as described except that 2D4 coated wells were saturated with 1 μg/ml of rfGPIbα (10).

VWF:RCo in plasma spiked with purified VWF

The ELISA was performed as described in the VWF:RCo ELISA using plasma glycocalicin except that to the 1/32 dilution of plasma pool (final volume of 500 μl) either no purified VWF or 6.6, 13.2 or 19.8 mU purified VWF was added in the presence of a constant amount of glycocalicin. Next, 200 μl of the above solution was applied to the first well and then ½ dilutions were performed. The ristocetin-induced VWF-binding was further performed as described above.

The VWF:RCo of purified VWF was determined using the normal plasma pool as a reference.

Competition ELISAs

To study the competition between VWF and anti-GPIb MAbs for the binding to plasma glycocalicin, the ELISA was performed as described for the VWF:RCo ELISA except that a dilution series of the inhibitory anti-GPIbα MAbs was added to the wells containing the captured glycocalicin for 30 min at RT. Next, a solution containing plasma pool (final dilution 1/32) and ristocetin (final concentration 1 mg/ml) were added for another hour at 37°C. Bound VWF was detected with anti-VWF-Ig-HRP.

Affinity chromatography

Plasma was depleted of glycocalicin using affinity chromatography. The anti-GPIb MAb 24B3 was coupled to CNBr-activated Sepharose® 4B (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. The column was equilibrated with TBS, 0.4 mM CHAPS (Boehringer Mannheim, Mannheim, Germany) and normal plasma pool containing 1 mM N-ethylmaleimide (ICN, Ohio, USA), 1 mM leupeptin (Sigma) and 1 mM PMSF (Sigma) was applied on the column. Plasma depleted of glycocalicin was collected in the flow through. The column was regenerated using 3 cycles of washing with 0.1 M glycine, pH 2.8 followed by TBS. The presence of residual glycocalicin in the flow through was determined by ELISA as described for the capturing of glycocalicin on anti-GPIb MAbs. To check whether VWF levels remained unchanged after affinity chromatography a VWF:Ag ELISA was performed as described (10).

Results

VWF:RCo ELISA using plasma glycocalicin instead of rfGPIbα as a GPIbα source

To investigate whether glycocalicin present in plasma could be an alternative source for rfGPIbα in the VWF:RCo ELISA, we have attempted to use non-purified plasma glycocalicin by capturing it from human plasma using anti-GPIbα Mabs. We screened 42 anti-GPIbα MAbs to determine whether any of these could capture plasma glycocalicin and support the ristocetin-induced VWF-binding. Of the 42 MAbs screened, 27 MAbs were able to capture glycocalicin directly from plasma, albeit with different affinities (not shown). However, only one, i.e. MAb 24B3, could capture plasma glycocalicin in such a way that the ristocetin-induced VWF-binding to MAb 24B3-bound glycocalicin paralleled that found for 2D4-captured rfGPIbα (Fig. 1); thus reaching *A* (490 nm) values high enough to make a robust readout possible (*A* (490 nm) 0.83 ± 0.11, n=3). The same results were obtained using glycocalicin purified from outdated platelets (not shown). In contrast, when, for example, MAb 2D4, i.e. the antibody used to capture rfGPIbα in the reported ELISA, was used to capture plasma glycocalicin, only a low level of ristocetin-induced VWF-binding was observed (Fig. 1), resulting in *A* (490 nm) values not high enough for a robust readout (a 1/32 dilution of plasma pool resulted in plateau values of *A* (490 nm) = 0.37 ± 0.11, Fig. 1).

In conclusion, when presented by MAb 24B3, plasma glycocalicin appeared to be suited for a VWF:RCo assay and is a more practical alternative for the 2D4 captured rfGPIbα.

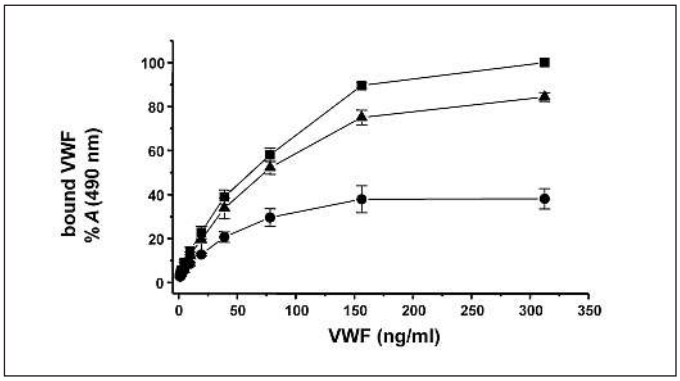

Figure 1: VWF:RCo determination in ELISA with either MAb 24B3-captured plasma glycocalicin or MAb 2D4-captured rfGPIbα. Wells were coated with either MAb 2D4 (●, ■) or 24B3 (▲) and were saturated with either a constant amount (1 µg/ml) of rfGPIbα (■) or plasma glycocalicin (½ plasma dilution) (●, ▲). VWF-binding to rfGPIbα and glycocalicin was studied in the presence of 760 µg/ml and 1 mg/ml ristocetin respectively. Different dilutions of a plasma pool were added and bound VWF was detected using anti-VWF-Ig-HRP. (n = 3, mean ± s_D, 100 % *A* (490 nm) values = 0.99 ± 0.16).

Optimization and validation of the VWF:RCo ELISA using plasma glycocalicin

Binding of VWF to 24B3 captured plasma glycocalicin in the presence of different ristocetin concentrations revealed that maximal VWF-binding occurs when induced by 1 mg/ml ristocetin (Fig. 2A). This ristocetin concentration was therefore used in all following experiments. Concentrations of 1.2 mg/ml and 1.5 mg/ml of ristocetin were also tested but resulted in too high background values. The VWF-binding was specific as no binding was observed in the absence of ristocetin or in the presence of the inhibitory anti-GPIb MAb 6B4 (Fig. 2B), but not of a control antibody.

To test whether the choice of plasma as a source of glycocalicin would influence the extent of the ristocetin-induced binding of VWF in this ELISA and thus the determination of the VWF:RCo, 19 different normal plasmas were selected to saturate 24B3 with glycocalicin. The ristocetin-induced VWF-binding was then studied using the normal plasma pool (as a source of VWF) in dilutions of 1/32 and 1/64. Figure 3A demonstrates that most normal plasmas can be used as a source of glycocalicin. The coefficient of variation (CV=(s_D/mean)*100) for the 1/32 and 1/64 dilution was 10.4 % (n=19) and 11 % (n=19) respectively.

Next, the influence of the presence of glycocalicin in the plasma used as VWF source in the VWF:RCo ELISA was studied. The binding of VWF present in normal plasma, i.e. containing glycocalicin, or in plasma depleted of glycocalicin, to 24B3 captured plasma glycocalicin was compared. Normal plasma was depleted of glycocalicin through affinity chromatography using an anti-GPIbα MAb. The absence of glycocalicin and a normal VWF concentration were both confirmed in ELISA. No major effect of the absence of glycocalicin was seen when a 1/32 dilution (or higher dilutions) of plasma as a source of VWF was tested (Fig. 3B), confirming that at the final plasma dilution tested, plasma glycocalicin does not compete with antibody bound glycocalicin for VWF-binding.

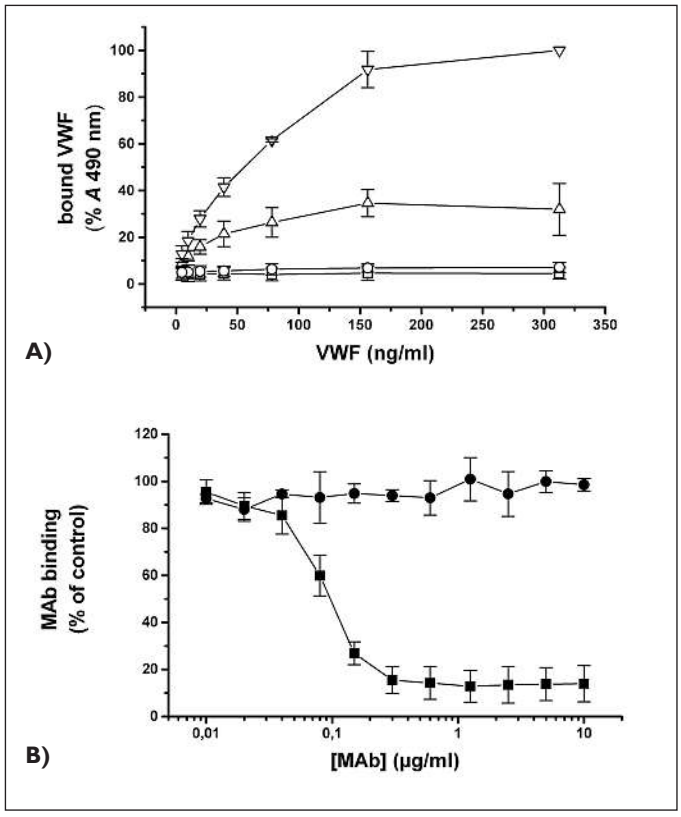

Figure 2: Sensitivity and selectivity of VWF-binding to captured plasma glycocalicin. (A) 24B3 coated wells were saturated with plasma glycocalicin by adding a constant amount of plasma and VWF-binding was performed as described under Figure 1 in the presence of 0 (□), 0.5 (○), 0.76 (△) and 1 mg/ml ristocetin (▽). (n = 3, mean ± s_D, 100 % *A* (490 nm) values were 1.11 ± 0.3). (B) The VWF:RCo ELISA was performed as described under A but increasing concentrations of the inhibiting anti-GPIb MAb 6B4 (■) or non-inhibiting anti-GPIb MAb 11A11 (●) were added to plasma glycocalicin saturated wells for 30 min before constant amounts of plasma pool as a source of VWF (final dilution 1/32) in the presence of 1 mg/ml ristocetin were added for another hour. (n = 3, mean ± s_D).

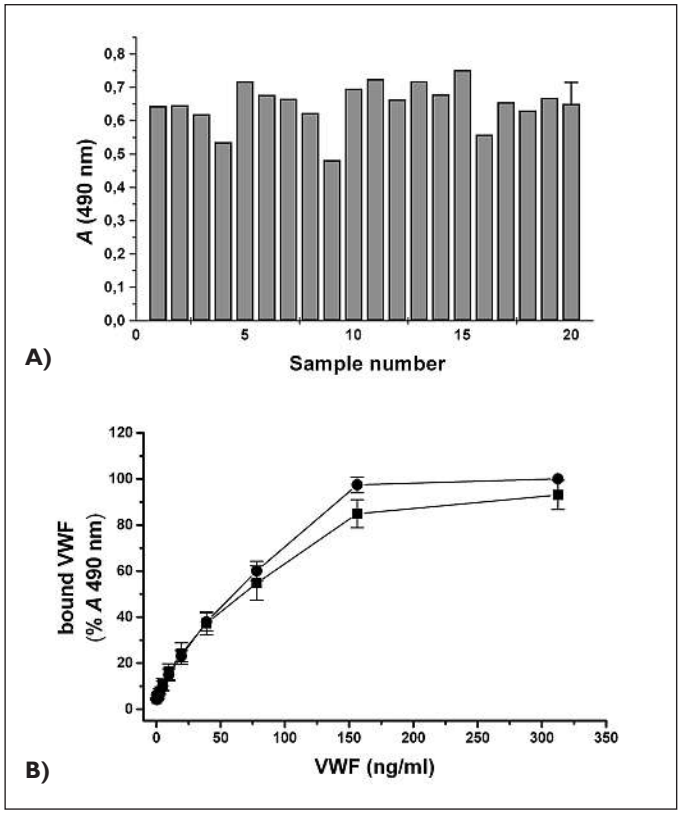

Figure 3: Variability of the plasma source of glycocalicin (A) and influence of the presence of glycocalicin in the plasma used as VWF source (B) in the VWF:RCo ELISA. (A) 24B3 coated wells were saturated with glycocalicin from 19 different plasmas (bar 1 to 19) by adding a constant amount of each plasma. Normal human plasma pool (as a source of VWF) was added in a 1/32 and 1/64 dilution in the presence of 1 mg/ml ristocetin. Bound VWF was detected using anti-VWF-Ig-HRP. Only *A* 490 nm values of 1/32 dilutions are shown. Last bar represents the mean ± s_D of the 19 samples tested. (B) Glycocalicin from plasma was captured by MAb 24B3 and dilution series starting from a 1/32 dilution of the normal plasma pool (●) or of plasma affinity depleted of glycocalicin (■) were added in the presence of 1 mg/ml of ristocetin. Bound VWF was detected with anti-VWF-Ig HRP. (n = 3, mean ± s_D, 100 % *A* (490 nm) values were 1.04 ± 0.29).

To then validate plasma glycocalicin for application in the VWF:RCo assay, the intra-assay variability was determined by testing 6 dilutions of plasma pool on 8 replicates in one run. The CV was less then 10% for each dilution point (6%, 5%, 9%, 7%, 5% and 7% respectively). The inter-assay variability was determined by testing the same plasma dilutions on six replicates over 3 days and by 2 different investigators. The maximum CV observed for each data point was 10%, 9%, 9%, 9% and 9.5% respectively. The detection limit (DL) and quantification limit (QL) were determined by assaying 9 dilutions (1/32 to 1/8192) of plasma pools in 3 different ELISAs, and each time the zero standard was obtained from a plasma of a type 3 VWD patient. The DL (defined as 3SD above the mean of zero standard) was 0.0005 U/ml. The QL, the minimum concentration that can be measured from assay to assay with CV < 20% was 0.0005 U/ml.

We also investigated whether VWF:RCo activities of more than 1U/ml could be correctly measured. We therefore spiked 16 mU (in 500 µl) of a normal plasma pool (1U/ml original activity) with 6.6 mU, 13.2 mU or 19.8 mU of purified VWF. Table 1 shows that the VWF:RCo in plasmas containing up to 228 % VWF:RCo activity when compared to the VWF:RCo present in a normal plasma pool, still can be accurately determined in this ELISA.

VWF:RCo in plasma from normal individuals and VWD patients

The VWF:RCo determined using the VWF:RCo ELISA with captured plasma glycocalicin was determined in plasma of 33 normal individuals and ranged from 0.46 U/ml to 2.7 U/ml (Fig. 4).

The VWF:RCo values ranged from 0.09 to 0.48 U/ml when measured in 19 type 1 VWD plasmas, from 0 to 0.4 U/ml in 16 type 2A patients, from 0 to 0.66 U/ml in 9 type 2B patients and from 0.01 to 0.16 U/ml in 8 type 2M patients. For the 7 type 3 patient plasmas, no VWF:RCo activity was detected (Fig. 4).

Table 1: Determination of VWF:RCo in plasmas containing up to 228% VWF:RCo. To 16 mU of normal plasma pool different mU of purified VWF were added, the final expected VWF:RCo was calculated and was next determined using the VWF:RCo ELISA.

mU VWF:RCo added to plasma pool	Calculated VWF:RCo (U/ml)	Measured VWF:RCo (U/ml)	CV (%) of measured VWF:RCo
6.6	1.45	1.75 ± 0.21	12
13.2	1.85	2.08 ± 0.12	6
19.8	2.28	2.75 ± 0.21	8

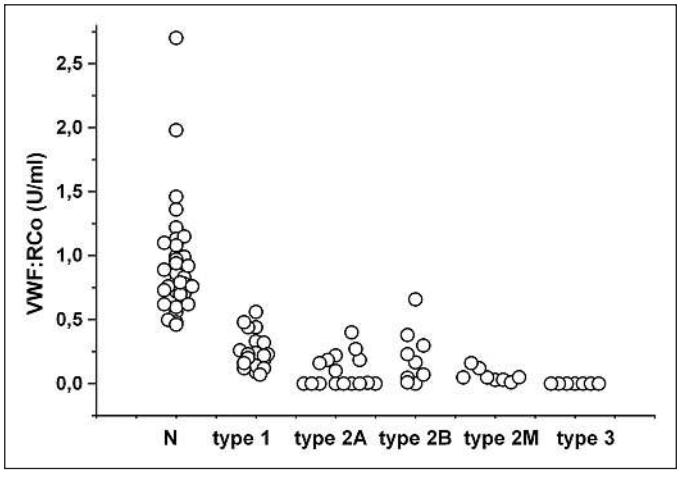

Figure 4: VWF:RCo levels from normal individuals and from VWD patients. Data points represent the VWF: RCo in the plasma from 33 normal individuals, 19 type 1 VWD patients, 16 type 2A, 9 type 2B, 8 type 2M and 7 type 3 VWD patients. Each data point represents the mean of the VWF:RCo obtained from 2 analysis of 2 different plasma dilutions (n = 4).

Comparison of the current ELISA with other methods

To compare the current ELISA where we use 24B3-captured plasma glycocalicin with our previous ELISA where 2D4-captured rfGPIbα is used to measure the ristocetin-induced VWF-binding, VWF:RCo was also determined using the latter test on 22 of the 33 normal plasmas and the values ranged from 0.34 U/ml to 0.99 U/ml. The VWF:RCo of most of the VWD plasmas (17 type 1, 10 type 2A, 1 type 2B and 3 type 3 VWD) have previously been determined in the VWF:RCo ELISA using the rfGPIbα (10). Figure 5 shows that there is a good correlation (r = 0.943) between the data obtained using either plasma glycocalicin or rfGPIbα as a source of GPIbα in the VWF:RCo ELISA.

The data obtained with the glycocalicin based VWF:RCo ELISA furthermore correlated with those previously obtained with the classical agglutination method, as a correlation of r = 0.963 was calculated (Fig. 6). Platelet agglutination data were determined at the Center for Molecular and Vascular Biology, KU Leuven, Leuven Belgium or at the 2nd Department of Medicine, University of Debrecen, Hungary and were available for 11 normal plasmas, 19 type 1, 16 type 2A, 9 type 2B, 8 type 2M and 7 type 3 plasmas.

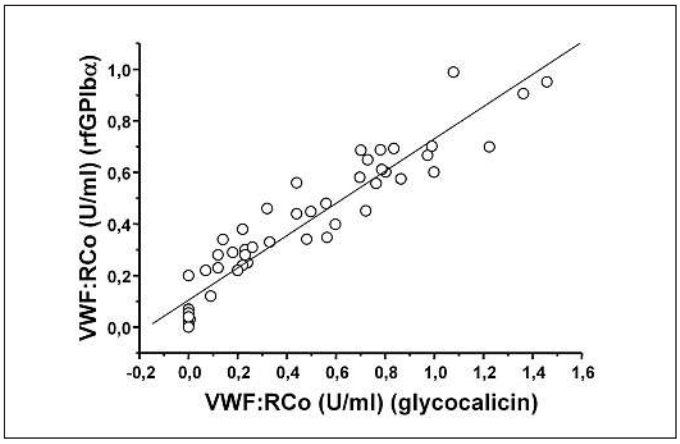

Figure 5: Scatter plot analysis of VWF:RCo determined in the VWF:RCo ELISA using plasma glycocalicin or rfGPIbα. VWF:RCo values were determined in the VWF:RCo ELISA using either 24B3 captured plasma glycocalicin or 2D4 captured rfGPIbα.

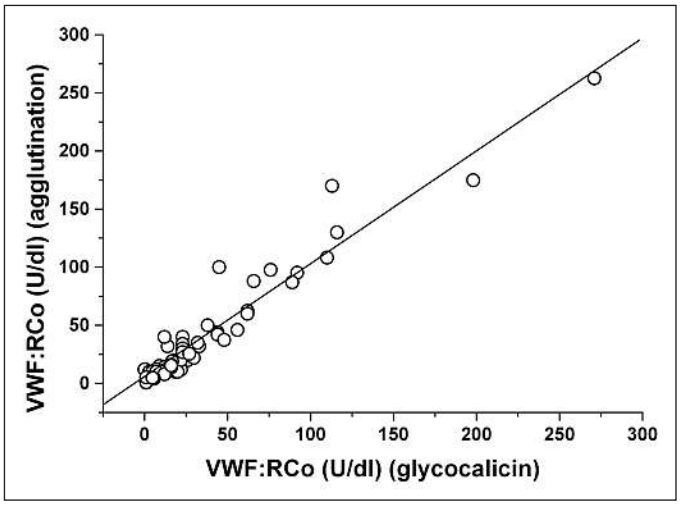

Figure 6: Scatter plot analysis of VWF:RCo determined in the VWF:RCo ELISA using plasma glycocalicin or in the platelet agglutination test. Values measured in the ELISA test using glycocalicin are the same as in Figure 4 and the values determined in the platelet agglutination test were obtained at the Center for Molecular and Vascular Biology, KU Leuven, Leuven Belgium or at the 2nd Department of Medicine, University of Debrecen, Hungary.

Discrimination between type 1 and type 2 VWD patients using the glycocalicin based VWF:RCo ELISA

Calculation of the VWF:RCo/VWF:Ag ratio allows a discrimination between normals, type 1 patients on the one hand and type 2 VWD patients on the other hand (24). VWF:RCo/VWF:Ag ratios < 0.7 classify the patients as type 2 VWD. Table 2 demonstrates that for all normal plasmas VWF:RCo/VWF:Ag ratios > 0.7 are obtained. Only one type 2 patient is incorrectly diagnosed using the glycocalicin based VWF:RCo ELISA, while for 10 type 2 patients VWF:RCo/VWF:Ag ratios < 0.7 were obtained. For the type 1 patients, 6 are incorrectly diagnosed with both the ELISA and the agglutination test, may be questioning the earlier typing. Of the other type 1 patients, 3 VWF:RCo/VWF:Ag ratios are < 0.7, when using the ELISA and 1 using the agglutination test.

Table 2: VWF:RCo/VWF:Ag ratios to discriminate between type 1 and type 2 patients. VWF:RCo was determined using either the VWF:RCo ELISA based on 24B3-captured plasma glycocalicin (ELISA) or the platelet agglutination test (agglutination). VWF:RCo/VWF:Ag < 0.7 should identify type 2 patients. Data in bold are not in agreement with this ratio, and thus might result in misdiagnosis of VWD patients.

normals		Type 2A	
ELISA	**agglutination**	**ELISA**	**agglutination**
0.9	1.35	0.11	**1.33**
0.9	2.0	0.02	0.27
0.9	ND	0.23	0.13
0.94	1.06	0.09	**1.12**
0.79	1.02	0.04	0.44
1.05	1.09	0.1	**1.22**
0.93	0.91	0.06	**0.75**
0.99	0.98	0.04	**0.75**
0.98	0.99	0.07	**0.8**
1.07	0.95	0.05	0.57
0.89	0.86	0.22	0.22
1.22	1.18	0.48	0.26
		0.4	0.21
		0.32	0.16
		0.4	0.34
		0.56	0.52
Type 1		**Type 2B**	
ELISA	**agglutination**	**ELISA**	**agglutination**
0.56	**0.63**	0.03	0.38
1.14	0.98	0.13	0.28
1.19	1.06	0.43	0.32
0.97	**0.51**	0.43	0.56
0.59	1.02	0.63	**0.85**
1.02	1.09	0.49	0.49
0.8	0.76	0.19	0.28
0.72	1.1	0.02	0.12
0.45	0.75	0.5	0.59
0.68	**0.53**		
0.26	**0.59**	**Type 2M**	
0.32	**0.38**	**ELISA**	**agglutination**
0.48	0.71	0.28	**0.96**
0.43	1.43	0.22	**0.72**
0.46	**0.42**	0.36	**0.75**
0.26	**0.44**	0.12	0.39
0.88	1.15	**0.75**	0.5
0.73	0.7	0.38	0.28
0.92	0.72	0.27	0.27
		0.56	0.56

Discussion

We have recently developed and validated an ELISA to determine the VWF:RCo (10, 11) and demonstrated that this test largely overcomes the drawbacks of the routinely used agglutination test, one of the important tests used in the diagnosis of VWD patients. The advantages of this test were recently confirmed by Federici et al. (25). In this ELISA a rfGPIbα is used, expressed by CHO cells, to measure the ristocetin-induced binding of VWF. However, in order to facilitate the accessibility of this ELISA to the clinical laboratory, an alternative for the rfGPIbα is needed, as the production and purification of rfGPIbα is cumbersome and expensive. We reasoned that glycocalicin could be a good alternative. Two sources of glycocalicin were of interest: either produced by proteolytic digestion of the platelet receptor GPIb/V/XI complex (12, 23) or selected from plasma itself, known to contain glycocalicin levels around 1–3 μg/ml in normal individuals (17, 18). The latter seemed preferable in view of the possibility to capture glycocalicin directly from the plasma without the need for further purification. Twenty seven of the 42 screened anti-GPIbα MAbs were able to capture glycocalicin from plasma. However, only MAb 24B3 was able to efficiently capture glycocalicin to enable ristocetin-induced VWF-binding. The resulting ristocetin-induced VWF-binding to 24B3-bound plasma glycocalicin was specific, as no binding was observed in the absence of ristocetin and since VWF-binding could be inhibited by function blocking anti-GPIb MAbs.

The VWF:RCo determined in the glycocalicin-based ELISA correlated well with the VWF:RCo determined in the platelet agglutination test (r = 0.963) and in the rfGPIbα-based ELISA assay (r = 0.943). Determination of ratio of VWF:RCo to VWF:Ag helps distinguishing type 1 and type 2 VWD patients as low ratios (<0.7) identify disfunctional type 2 VWF. Combination of the VWF:RCo determined in the glycocalicin based ELISA and the VWF:Ag allows the identification of type 1 and type 2 VWD patients (Table 2). The glycocalicin-based VWF:RCo ELISA performs better than the aglutination test in identifying type 2 patients. The latter is probably due to the low sensitivity of the agglutination test.

Validation of the plasma glycocalicin-based test revealed that this ELISA has the same advantages over the currently used agglutination test as has the rfGPIbα-based ELISA. The test has a low inter-assay and intra-assay variability (CV< 11%) and can be readily automated. Although theoretically rfGPIbα is a more reproducible and constant source of GPIb than plasma glycocalicin, pooled human plasma nevertheless remains a better and more reproducible source of GPIb than human platelets and clearly more readily available than rfGPIbα. We demonstrated that almost any normal plasma can be used to capture glycocalicin with no major differences in the determination of VWF:RCo in test samples. The end point of the test is colorimetric and is more easily to evaluate then the aggregation curves in the agglutination test, the ELISA is sensitive with a QL of 0.0005 U/ml and minimal amounts of patient plasma are required (8 μl) making the test ideal for use in pediatrics.

In conclusion, we have demonstrated that in the VWF:RCo ELISA, instead of rfGPIbα, plasma glycocalicin can be captured directly from plasma using the appropriate anti-GPIb MAb. Our MAb 24B3 is freely available for use in other research laboratories to allow a multicenter evaluation in the determination of the VWF:RCo.

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