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von Willebrand factor contained in a high purity FVIII concentrate (Fanhdi®) binds to platelet glycoproteins and supports platelet adhesion to subendothelium under flow conditions

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ABSTRACT

Background and Objective. There is evidence suggesting that von Willebrand factor (VWF) from high purity factor VIII concentrates could be of clinical use in the management of patients suffering from VWD. We analyzed structural and functional characteristics of VWF present in a high purity factor VIII concentrate VWF_{HPC} (Fanhdi®). The multimeric structure, the ability to bind to platelet GP Ib/IX or GP IIb/IIIa, and the capacity of VWF_{HPC} to promote platelet adhesion on injured vessels were investigated and compared with that present in standard plasma cryoprecipitates [VWF_{CRYO}].

Design and Methods. Binding studies were carried out by incubating radiolabeled VWF and washed platelets, which were activated with either ristocetin (1 mg/mL; for GP Ib/IX), or thrombin (2.5 U/mL; for GP IIb/IIIa). Platelet adhesion was assessed in a perfusion system (shear rate = 800 s-1, 10 min) in which the source of VWF was added (at 0.4 or 0.8 U/mL VWF:Ag) to washed platelets and red cells suspended in a human albumin solution. The deposition of platelets onto the perfused subendothelial surface was morphometrically evaluated and expressed as percentage of surface coverage (%SC).

Results. The VWF_{HPC} (152 Units VWF:RCof/mg protein; VWF:RCof/VWF:Ag = 0.97), lacked only a small proportion of high-molecular-weight multimers present in VWF_{CRYO}. Binding affinities (Kd values, nM) of VWF_{HPC} were similar to those of VWF_{CRYO} (5.3±0.86 vs 5.2±0.95, for GP Ib/IX; and 11.6±2.7 vs 15.4±1.7 for GPIIb-IIIa). A slightly, though not significantly, higher binding capacity for these receptors (Bmax values, molecules/plt) was obtained for VWF_{HPC}. The %SC in perfusions in the presence of albumin was < 10%. Addition of VWFHPC or VWF_{CRYO} significantly increased the %SC, with values of 27.1±4.9 and 17.5±2.8%, respectively with 0.4 U/mL ($p<0.004$ and $p<0.02$ vs albumin); and 30.8± 4.9% and 20.03±4.1%, respectively, at 0.8 U/mL ($p<0.001$ and $p<0.02$ vs albumin).

Interpretation and Conclusions. Our data show that VWF present in the high purity FVIII concentrate Fanhdi® retains the functional capacity to bind to GPs Ib/IX and IIb/IIIa and to promote platelet adhesion onto exposed subendothelium.

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Von Willebrand's factor (VWF) is a plasma adhesive protein which is quantitatively or qualitatively deficient in von Willebrand's disease (VWD). This adhesive protein binds to vessel subendothelium and to a platelet receptor located in the glycoprotein complex Ib/IX (GP Ib/IX), thus mediating the initial attachment of platelets onto damaged vascular areas.[1-3] Cryoprecipitates have been traditionally used in the substitutive treatment of von Willebrand's disease.[4-6] Experimental and clinical studies have demonstrated that the VWF contained in cryoprecipitates improves the platelet adhesion defect of VWD patients.[7-9]

Commercially available antihemophilic concentrates containing VWF which were manufactured in the past decades were not useful in the treatment of bleeding episodes in vWD patients.[10-12] It is very likely that ultrastructural abnormalities of VWF contained in very early preparations accounted for its inability to support platelet adhesion.[7] The improvement of technology has facilitated the preservation of the VWF:Ag, RiCof activity and multimeric pattern in a series of FVIII concentrates.[13,14]

In the last decade, intermediate purity FVIII concentrates containing VWF have proven to be therapeutically useful in the prevention and control of hemorrhagic episodes in VWF deficient patients.[4,15] Clinical studies have suggested that the VWF present in high purity FVIII concentrates could improve clinical hemostasis in VWD patients.[16,17] It remains to be established whether functional characteristics of VWF could also be preserved during industrial processes used for the preparation of high purity FVIII concentrates.

In the present study, we evaluated the ability of VWF present in a high purity factor VIII concentrate [VWF_{HPC}] (Fanhdi®, Instituto Grifols S.A., Barcelona, Spain) to bind to platelets and to support platelet adhesion. The capacity of VWF_{HPC} to promote platelet adhesion on injured vessels was investigated in a well established perfusion system[18-20] using arterial blood flow conditions. For the latter purpose, perfusates consisting of isolated platelet suspensions, albumin and washed red blood cells, were incubated with VWF_{HPC}, at final concentrations of 0, 0.4 or 0.8 IU VWF/mL. The interaction of platelets with the perfused damaged subendothelium was morphometrically evaluated. Results of these studies were compared in all cases with those obtained in experiments with VWF obtained from standard blood bank cryoprecipitates (VWF_{CRYO}).

Design and Methods

Characteristics of VWF sources

Standard single donor cryoprecipitates,[21] locally produced at the study centers, were added to reconstituted blood samples. The cryoprecipitates were assumed to contain an average amount of 4 IU VWF/mL, as determined by periodic quality testing in the blood bank.

Vials of the high purity factor VIII concentrate (Fanhdi®, Instituto Grifols S.A., Barcelona, Spain) were dissolved in sterile water immediately before use.

Purification of VWF

Normal VWF, employed as the control for the glycoprotein binding studies, was isolated and purified from a pool of blood bank cryoprecipitates (VWF_{CRYO}) as described elsewhere.[22] Fanhdi® was purified according to the process described by Ristol *et al.*[23] For the glycoprotein binding assays, the VWF in Fanhdi® (VWF_{HPC}) was further purified in a Sepharose CL4B column, to remove albumin added as stabilizer and trace contaminant amounts of fibrinogen and fibronectin.

Assessment of FVIII/VWF related activities and structure

Coagulant factor VIII activity (FVIII:C) was measured by the one stage clotting time.[24] Ristocetin cofactor activity (RiCof) was measured by using formaldehyde-fixed platelets.[25] Values were expressed in IU/mL with reference to a plasma calibrated against the 2nd international standard for FVIII related activities in plasma (87/718, National Institute for Biological Standards and Controls, Potters Bar, UK). von Willebrand factor antigen (VWF:Ag) was measured by enzyme immunoassay.[26] The VWF multimeric structure was analyzed by sodium dodecyl sulphate (SDS)-agarose gel electrophoresis followed by electrotransfer to PVDF membranes.[27]

Blood collection and platelet isolation

A standard unit of blood was obtained from healthy individuals who had not ingested drugs affecting platelet function during the previous 10 days. All healthy individuals passed a physical examination and detailed medical history, according to the guidelines of the American Association of Blood Banks,[21] and gave informed consent in accordance with the current version of the Declaration of Helsinki. Blood was anticoagulated with citrate-phosphate dextrose (CPD-final concentration of citrate in blood 19 mM).

Each blood donation (450 mL) was immediately separated into its main components: packaged red blood cells (RBC), platelet rich plasma (PRP) and platelet poor plasma (PPP). Platelets were isolated from PRP and washed following a centrifugation-suspension method previously described.[28]

Binding studies

VWF_{CRYO} and VWF_{HPC} to be used in binding assays were radiolabeled with carrier-free Na-^{125}I (Amersham International, England) using Iodogen (Pierce Chemical, Rockford, USA), as described by Fraker and Speck.[29] Specific activities ranged between 0.3 and 0.8 mCi/mg of protein. Polyacrylamide gel electrophoresis of radiolabeled proteins showed no structural alteration as compared to the unlabeled counterparts.

Binding assays of VWF to platelets were performed essentially as previously described.[22] Briefly, washed platelets (1×10^8 cells/mL) were incubated with increasing concentrations of ^{125}I-VWF (0-20 µg/mL). Binding to GP Ib/IX was induced with ristocetin (1 mg/mL), whereas binding to GP IIb/IIIa was measured following platelet activation with α-thrombin (2.5 NIH U/mL, 5 min), the activating effects being arrested by addition of a 20-fold excess of hirudin. Incubations were performed without agitation at room temperature for 30 min, after which platelet-bound and free ligand were separated by centrifugation and counted (LKB, Multigamma, Pharmacia, Sweden). Standard binding parameters: a) Bmax: total concentration of binding sites; b) Kd: dissociation constant, i.e. the concentration of free ligand at which the binding sites are half-saturated with ligand; and c) NSB: nonspecific, nonsaturable binding, were derived from Scatchard type analysis using the computer-assisted program Ligand.[30]

Preparation of perfusates

von Willebrand factor depleted perfusates were produced by suspending RBC (to a 40% volume) and washed platelets (to raise platelet count to 2.0×10^8/mL) into an adequate volume of a plasma substitute made up of 4% albumin (w/v) plus 2.5 mM Ca^{2+}.[3] One unit of whole blood provided enough perfusates to run a complete set of experiments (5 perfusions). Amounts of VWF_{HPC}, or VWF_{CRYO} were calculated to give rise to theoretical concentrations equivalent to 0.4 or 0.8 IU VWF/mL.

Perfusates were incubated for 30 min in a water bath at 37°C prior to the perfusion experiments.

Samples of this reconstituted blood were used for determination of hematocrit, platelet count, arachidonic acid induced platelet aggregation, FVIII:C, RiCof and VWF:Ag.

Blood perfusions and morphometry

The evaluation of platelet deposition on subendothelium was performed as previously reported.[31,32] Abdominal aortas obtained from New Zealand white rabbits of 2.5 kg in weight were everted and enzymatically denuded.[3] Perfusion experiments were performed at 37°C in perfusion chambers.[31] Flow was obtained by pumping the blood through a hemodialysis blood pump (Renal Systems, Minneapolis, Minn., USA) at the appropriate flow rates to produce a wall shear rate of 800 s-1. After 5 min perfusion, the segments were rinsed with phosphate buffered saline (PBS), fixed with glutaraldehyde, embedded in JB-4 plastic compound (Polysciences, Warrington, Pa, USA), thin sectioned for light microscopy, and stained with methylene blue.[33] Platelet interaction with subendothelium (SE) was morphometrically evaluated.[31] Using a specially developed computer program.[34] the interaction of platelets with the subendothelium was evaluated in 20 different microscope fields and expressed as percentage surface coverage (%SC).

Statistical analysis

The results of the experiments were expressed as mean ± SEM. Statistical differences in morphometric data and in binding parameters were assessed by the Student's test. The level of statistical significance was established at $p < 0.05$.

Results

Activity and structure of VWF

The VWF_{CRYO} used as control for the glycoprotein binding studies had a ristocetin cofactor activity (RiCof) of more than 100 U/mg of protein, and a multimeric structure similar to that of plasma. The VWF_{HPC} did not show alterations of the multimeric structure as a consequence of the purification process (Figure 1). The purified VWF had a RiCof of 152 U/mg with a RiCof/VWF:Ag = 0.97. Fibrinogen, fibronectin and albumin were undetectable by nephelometry in the purified material (data not shown).

Binding of VWF to platelets

The ability of VWF_{HPC} to interact with the platelet receptors GP Ib/IX and GP IIb/IIIa was compared with that of VWF_{CRYO} in radioligand binding experiments. As summarized in Table 1, Scatchard analysis of isotherms demonstrated that both preparations bind to these receptors with similar affinities (Kd values). For either GP Ib/IX or GP IIb-IIIa, VWFHPC bound with slightly higher capacity (Bmax values) than VWF_{CRYO}, although differences never reached the levels of statistical significance.

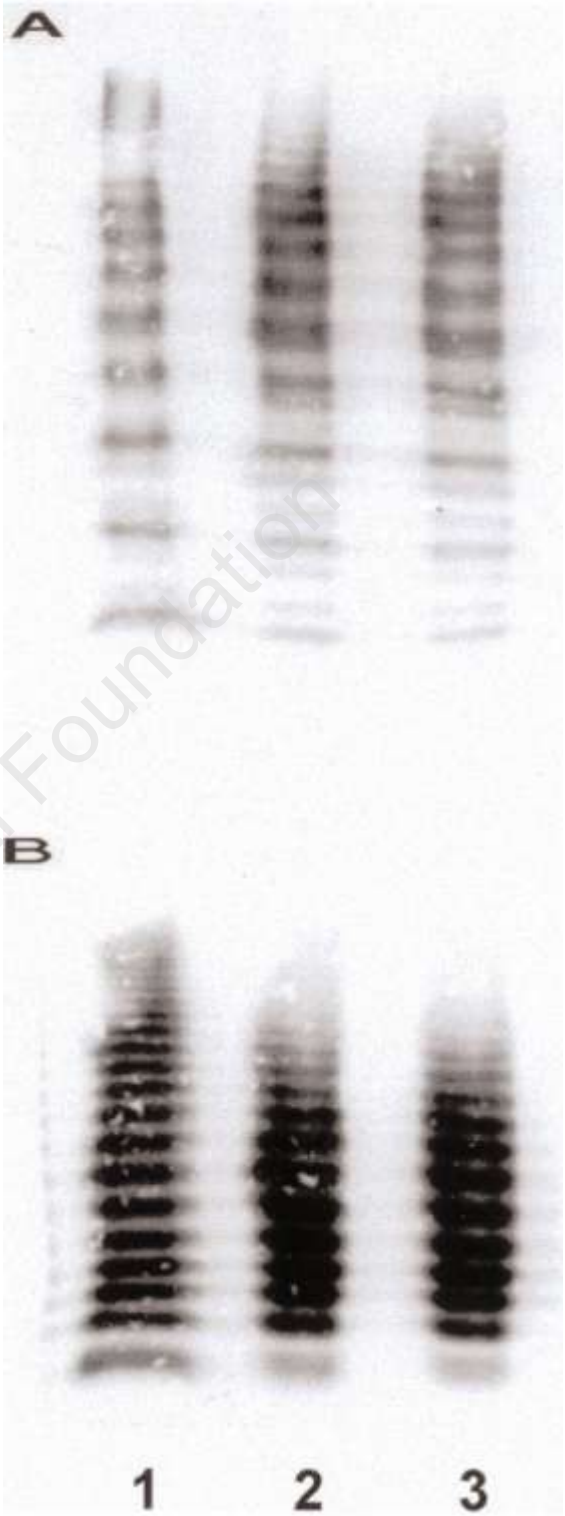

Figure 1. Multimeric patterns for control von Willebrand factor (VWF_{CRYO}) (lane 1) and for the VWF_{HCP} present in the high purity FVIII concentrate Fanhdi® before (lane 2), and after purification (lane 3). The upper (A) and lower (B) panels correspond to high (2.2% agarose) and low (1.4% agarose) resolution gels, respectively.

Table 1. Binding of von Willebrand factor purified from cryoprecipitate (VWF_{CRYO}) or from a high purity concentrate (VWF_{HPC}) to GP Ib/IX and GP IIb/IIIa complexes.

	Binding to GP Ib/IX (n=11)		Binding to GP IIb/IIIa (n=5)	
VWF	Kd	Bmax	Kd	Bmax
VWF_{CRYO}	5.3±0.86	18,094±3,137	11.6±2.7	4,336±945
VWF_{HPC}	5.2±0.95	38,004±6,305	15.4±1.7	9,293±1,680

Mean ± SEM; Kd (nM); Bmax (molecules/platelet).

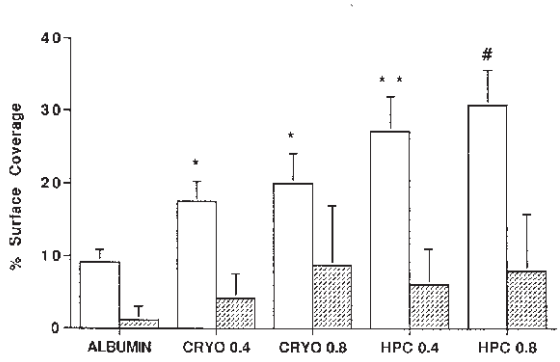

*Figure 2. Bar diagrams represent morphometric parameters obtained in perfusion studies. Bars express total percentage of vessel wall covered by platelets (empty bars) and percentage covered by aggregates of more than 5 µm (dashed bars) in experiments using cryoprecipitate [CRYO] or the high purity FVIII concentrate [HPC] as a source of von Willebrand factor. Each bar represents values ± SD (n=9). Marks denote *p<0.02, **p<0.004, and # p<0.001 vs albumin.*

Platelet adhesion studies

The surface covered by platelets in control perfusion studies performed in the presence of 4% albumin without any external source of VWF, reached values of 9.07±1.83%. The VWF:Ag levels in these perfusates containing only human albumin were always below 0.05 U/mL.

The VWF_{CRYO} caused a marked increase in the deposition of platelets onto the perfused damaged vessel (Figure 2). The morphometric evaluation of perfusions containing cryoprecipitate revealed percentages of surface coverage (%SC) of 17.5±2.8 and 20.03±4.11%, respectively for the theoretical concentrations of 0.4 and 0.8 U/mL. The increase in platelet coverage obtained was statistically significant with respect to that observed in studies with albumin alone ($p<0.02$).

The preparation of VWF_{HPC} also supported platelet adhesion. Surface coverage by platelets improved significantly (Figure 2). Average values of %SC reached values of 27.1±4.9 and 30.8±4.9%, respectively for the theoretical concentrations of 0.4 and 0.8 U/mL ($p<0.004$ and $p<0.001$ vs albumin).

Figure 3 illustrates morphologic differences between results obtained with the different preparations and concentrations tested in our studies. Platelet masses tended to be slightly more pronounced into the vessel lumen in studies with VWF_{CRYO} than in studies with VWF_{HPC}.

Discussion

The results of the present study demonstrates that the VWF contained in a high purity factor VIII concentrate retains a reasonably well preserved multimeric structure, binds to platelet GP Ib/IX and GP IIb/IIIa and corrects platelet adhesion to suben-

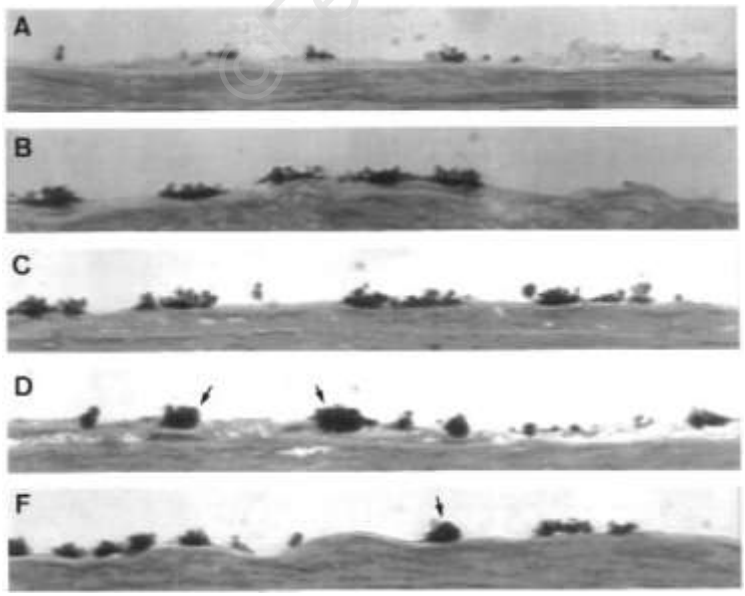

Figure 3. Light micrographs from sections representative of perfusions with 4% albumin (A), VWF_{HPC} at 0.4 and 0.8 U/mL (B, C), and VWF_{CRYO} at 0.4 and 0.8 U/mL (D, E). The von Willebrand factor present in both preparations improved platelet adhesion. The aggregates formed in the presence of cryoprecipitate were more prominent (arrows) than those formed with the high purity preparation (×600).

dothelial structures in perfusion studies *in vitro*.

Several clinical studies have demonstrated that VWF contained in intermediate purity concentrates are effective in the substitutive treatment of von Willebrand's disease.[7-9] It is evident that the improvement of purification technology has facilitated the preservation of functional abilities in the VWF which were not preserved with earlier industrial processes.[10-12] A previous experimental study demonstrated that VWF present in an intermediate purity FVIII concentrate (Haemate-P), was capable of binding to platelets and of supporting platelet adhesion to vascular subendothelium under flow conditions.[35] Results of the present study suggest that the latter functional characteristics can also be preserved in the VWF present in high purity FVIII concentrates.

Studies performed with *in vitro* perfusion devices have played a critical role in the understanding of platelet physiology. Thanks to these studies it is well established that binding of VWF to subendothelium and to platelet GP Ib/IX is of critical importance for platelet attachment onto damaged vascular surfaces.[2,3] Binding of VWF bound to the subendothelium with platelet GP IIb/IIIa mediates further platelet spreading onto the exposed vascular surface.[36,37] Interactions of platelet GP IIb/IIIa with plasma fibrinogen play a critical role in platelet-platelet interactions necessary for platelet aggregate formation[36] and growth.[37]

Data from the present study indicate that the VWF present in the high purity concentrate investigated (VWF_{HPC}) not only binds to GP Ib/IX and to GP IIb/IIIa in activated platelet suspensions, but also supports platelet attachment and spreading in studies in which platelets interact with damaged vascular surfaces under flow conditions. The ability of VWF_{HPC} to support platelet adhesion in our *in vitro* experiments was similar to that observed in experiments using VWF from cryoprecipitates or even slightly superior. In contrast, formation of aggregates seemed better preserved in perfusion experiments performed with VWF_{CRYO}. The reason for this apparent contrast might be explained by the different purity of both sources of VWF.[35] While VWF_{HPC} contains only VWF, the VWF in the cryoprecipitate is contaminated with other adhesive proteins such as fibrinogen which would facilitate platelet recruitment into aggregates and subsequent impairment in platelet adhesion.[38]

The presence of VWF in FVIII concentrates was initially thought to add stability to the coagulation factor. The VWF promotes association of light and heavy chains of FVIII thus protecting this factor against inactivation by activated protein C.[39,40] Average half lives of recombinant FVIII transfusion preparations seemed to be dependent on the pre-transfusional VWF:Ag levels.[41] Apart from these stabilizing actions, VWF in high purity FVIII concentrates could be potentially useful in the substitutive treatment of VWD patients.[42]

In summary, our present data support the idea that the purification process of VWF_{HPC} preserves functional activities of this adhesive protein. This might offer a good starting process to develop a specific concentrate of VWF for the treatment of von Willebrand's disease.

Contributions and Acknowledgments

JR and GE carried out all binding assays and adhesion experiments, respectively. RC and MIB performed the purification and characterization of VWF from FVIII concentrate Fanhdi®. JR, GE, JIJ and VV, contributed to the conception and design of the study, and took part in the interpretation of data and in the writing of the paper. RC and AO contributed with the analysis and interpretation of the results, and gave final approval of the version to be published. We thank Mrs. Montserat Viñas for her technical support.

The criteria for the order in which the names of the authors appear are based on their contribution to the design, analysis, interpretation of data and execution of the study.

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Disclosures

Conflict of interest: Instituto Grifols SA (RC, MIB and JIJ) produce FVIII concentrates for commercial purposes. Grifols SA provided the VWF_{HPC} used in the study free of charge, but no financial support.

Redundant publications: a companion paper appeared recently in this journal (ref. #42).

Manuscript processing

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