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Native multimer analysis of plasma and platelet von Willebrand factor compared to denaturing separation: Implication for the interpretation of satellite bands

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1 INTRODUCTION

Von Willebrand factor (vWF) is a large, multimeric glycoprotein which mediates platelet adhesion to the subendothelium in primary hemostasis, leading to aggregation and formation of platelet plugs at the site of the vascular injury (Weiss 1991). The glycoprotein therefore has a bridging function between platelet receptors, collagen, heparin and coagulation factor VIII. In addition, it stabilizes factor VIII during secondary hemostasis by acting as a carrier and protector for it (Brinkhous et al. 1985).

The protein circulates in human plasma at concentrations of between 8 and 25 mg/mL (Kao et al. 1981) with a half-life of 8 to 12 hours (Borchiellini et al. 1996), as a series of heterogeneous multimers ranging in size from about 500 kDa to over 20000 kDa (Titani et al. 1986).

vWF is synthesized in vascular endothelial cells (Jaffe et al. 1975) and megakaryocytes (Sporn et al. 1985), and is secreted into plasma after post-translational processing, like glycosylation, disulfide-bond formation and proteolytic cleavage. ADAMTS13, also known as von Willebrand factor-cleaving protease, is a metalloprotease, which degrades physiologically very large multimers of the protein. Deficiency of the metalloprotease is associated with the pathologic microangiopathy of thrombotic thrombocytopenic purpura (TTP) (Furlan and Lämmle 2001). vWF can be stored in Weible Palade bodies of endothelial cells as well as in the alpha granules of platelets (Wagner 1990).

Qualitative and/or quantitative abnormalities of vWF can be identified in patients who suffer from von Willebrand's disease (vWD), a common inherited bleeding disorder with an estimated prevalence of up to 1% (Montgomery and Kroner 2001, Sadler 1994, Sadler et al. 2000).

The disease was described by Erik von Willebrand, a physician from Finland, who reported a new type of bleeding disorder different from hemophilia for the first time. It exhibits mucocutaneous bleeding, an autosomal inheritance, a normal clotting time and a prolonged bleeding time by the Duke method (von Willebrand 1999). Hemophilia, in contrast, is characterized by internal bleeding, like hematomas and hemarthrosis, an X-linked recessive inheritance, a prolonged clotting time and a normal Duke bleeding time.

The major symptoms of vWD-affected individuals are: postoperative bleeding, epistaxis, menorrhagia, excessive bleeding after tooth extraction and soft-tissue bleeding (Sramek et al. 1995, Halimeh 2012). Nevertheless, the majority of these patients have no or mild

symptoms (Alesci et al. 2011). Gill et al. described in 1987, that symptoms are more apparent in vWD patients with the blood type 0.

The prognosis for vWD is generally good, and most individuals have a normal life expectancy. However, the prognosis can depend on an accurate diagnosis and an appropriate medical treatment.

vWD can be classified into three types, depending upon whether the individual defect is quantitative (types 1 and 3) and/or qualitative (types 2 and 3). The classification is based on criteria developed by the vWF Subcommittee of the International Society on Thrombosis and Hemostasis (ISTH), first published in 1994 and revised in 2006 (Sadler 1994, Sadler et al. 2006). The designation of vWD, caused by mutations within the vWF gene, was dropped from the 2006 criteria. Before the 1994 publication, vWD types were classified using Roman numerals.

There exist inherited and acquired forms of vWD. Most cases of vWD are hereditary, but abnormalities in the protein can also be acquired and are involved in a large number of other diseases (Sadler 1998), including thrombotic thrombocytopenic purpura (TTP) (Chapmann et al. 2012), Heyde's syndrome (Massyn and Kahn 2009, Vincentelli et al. 2003), and hemolytic uremic syndrome (Galbusera et al. 1999). Acquired vWD has also been described in patients with hypothyroidism and mesenchymal dysplasia, and in Wilm's tumour patients (Michiels et al. 2001, Shetty et al. 2011). vWD types 1 and 2 are usually inherited as autosomal dominant, whereas subtype 3 is usually inherited in an autosomal recessive pattern.

Type 1 is characterized by a quantitative loss of vWF. The amount of vWF is lower than normal and the level of factor VIII is also reduced. It is the most common type of the disorder (up to 70%) and it is one of the mildest forms of vWD. Most of the vWD type 1 cases are never diagnosed due to the asymptomatic and mild presentation.

vWD type 2, which accounts for approximately 10 to 30 percent of cases, is characterized by a qualitative defect of vWF and is further divided into subtypes 2A, 2B, 2M and 2N (Sadler et al. 2006). In Germany, the distribution of the subtypes was reported to be 74 % type 2A, 10 % type 2B, 13 % type 2M and 3.5 % type 2N (Budde et al. 2002). The amount of vWF is normal, but the multimer structure of the vWF is abnormal, or subgroups of large or small multimers are absent. The different subtypes 2M and 2N can be differentiated by more subtle alterations of the inner structure of smaller multimers (Furlan et al. 1993; Ruggeri and Zimmerman 1981; Ruggeri 1999). High-resolution agarose gels resolve the inner multimeric structure so that each smaller multimer dissociates into three

distinct subbands. These bands confirm the triplet structure, which consists of an intermediate band and two satellite bands (Aihara et al. 1986, Furlan et al. 1996, Siedlecki et al. 1996). The occurrence of satellite bands contributes currently to subclass identification.

The vWD type 2A is characterized by the absence of high molecular weight (HMW) multimers of vWF and by the fact that it has a disproportionately low ristocetin co-factor activity (vWF:RCo) compared to the von Willebrand's antigen (vWF:Ag).

Type 2B possesses an increased affinity of vWF for platelet glycoprotein Ib (GpIb), causing the absence of HMW, and it is associated with thrombocytopenia.

Type 2M (the M stands for “multimer”) includes variants with a defective interaction between vWF and platelets and may present an aberrant satellite structure in the multimer pattern but nevertheless exhibit no loss of HMW multimers.

The type 2N was defined (with “N” representing “Normandy” where the first individuals were identified) as a defect in the binding domain of vWF for the factor VIII, resulting a decrease of FVIII. The multimer structure in vWD type 2N is normal but the triplet structure can be aberrant.

Type 3 is the most severe form of vWD. These patients may have a total absence of vWF and factor VIII levels of often less than 10%, and therefore present with distinctive bleeding symptoms, like spontaneous soft-tissue bleeding, for example, hematomas and hemarthroses (Shamsakhzari et al. 2011).

Diagnosis of vWD is not only based on the vWF-multimer analyses by SDS-agarose electrophoresis, but also on the clinical history and measurement of the functional properties of the factor VIII/ vWF complex, including vWF antigen, ristocetin cofactor activity, factor VIII coagulant activity, ristocetin-induced platelet aggregation (RIPA) and vWF collagen-binding test (Budde et al. 2006; Nichols and Ginsburg 1997). At the moment, there is no genotypic classification of vWD available. More than 250 mutations of all vWD types have been identified (Keeney and Cumming 2001). The analysis of vWF multimers by electrophoretic separation and subsequent visualization is an important laboratory tool for distinguishing the subtypes of vWD. The choice of treatment depends essentially on the subtype of vWD, including the severity of the bleeding; therefore an adequate classification of the patient’s disease plays a key role in therapy.

The mainstay of treatment for patients with von Willebrand disease is the replacement of the deficient protein at the time when spontaneous bleeding occurs or before an invasive

surgical procedure (Mannucci 2004). Prophylaxis, in contrast to hemophilic patients, is not common, since the bleeding is less severe.

The FVIII deficiency, fibrin formation, defective platelet aggregation itself and subendothelial adhesion can be abolished by an increase of endogenous production of FVIII and vWF, using desmopressin or by replacement of plasma or vWF concentrates. Antifibrinolytic amino acids, platelet concentrates and oral estrogen-progesterone supplementation belong to adjuvant therapy.

Desmopressin is the treatment of choice for type 1 vWD, as the subtype has a functionally normal von Willebrand factor and desmopressin increases the endogenous release of FVIII and vWF (Mannucci et al. 1977), but it can also be used in some subtypes of type 2 vWD. Patients with vWD type 2, who secrete a qualitatively abnormal vWF, have an unsatisfying laboratory response to desmopressin (Ruggeri et al. 1982, Federici et al. 2004). In vWD type 2B it is generally contraindicated owing to a temporary thrombocytopenia after administering the drug (Holmberg et al. 1983). In vWD type 3 administration of desmopressin is ineffective (Ruggeri et al 1982).

Desmopressin (1-Desamino-8-D-Arginin-Vasopressin; DDAVP) is a synthetic substitute of vasopressin, also known as an antidiuretic hormone (ADH), a hormone that increases water absorption in the collecting ducts of the kidney. It is prescribed for treatment of diabetes insipidus and nocturia.

The advantages of using desmopressin as a therapeutic agent are its relatively low cost, unlimited availability and the important fact that there is no risk of infection, as there is with plasma substitutes.

The mechanism through which DDAVP stimulates the secretion of vWF from the Weibel-Palade bodies is cyclic adenosine monophosphate signaling (Kaufmann et al. 2000). Desmopressin is delivered intravenously at a dosage of 0.3 μg per kilogram of body weight, after a test dose has shown that the factor VIII and vWF rise adequately. An increase of about three to five times the initial value of vWF and factor VIII can be observed within thirty to sixty 60 minutes (Mannucci et al. 1977, 1981, 1992). It can also be applied by nasal inhalation at a single-dose of 150 μg in children and 300 μg in adults. Desmopressin also has some adverse effects. For example, it should not be used in patients with coronary heart disease (Bond and Bevan 1988, Byrnes et al. 1988) because it may cause myocardial infarction due to the secretion of ultra large vWF by endothelial cells, which provoke thrombogenesis (Moake et al. 1986).

In vWF patients in whom DDAVP is ineffective or contraindicated, the hemostatic defect can be corrected by replacement of factor VIII and vWF. Substitutes like fresh frozen plasma are not very efficient because they may cause a volume overload to gain hemostatic concentrations. Cryoprecipitate contains both factors at higher concentrations than fresh frozen plasma and minimizes the risk of viral transmission through the viral inactivation of the product.

There are a variety of commercial concentrates which contain a different concentration of the antihemophilic and von Willebrand factors. Wilfactin, for example, is a very high purity human von Willebrand factor concentrate which contains a small amount of factor VIII, indicated to stop and prevent bleeding in severe von Willebrand disease patients, like those with type 3 (Menache et al. 1996, Goudemand et al. 1998). Substitution of purified factor VIII only is not efficient for vWD therapy because, through the lack of the vWF, the antihemophilic factor possesses a very short half-life (Morfini et al. 1993).

Mucosal bleeding such as epistaxis and menorrhagia in vWD patients is in part due to the rich fibrinolytic activity of mucosal tracts (Mannucci 2004). Antifibrinolytic amino acids, which help to stabilize clots, are therefore a therapeutic strategy in patients with vWD, as well as in other inherited bleeding disorders (Mannucci 1998). It is more suitable as an adjuvant therapy together with desmopressin or factor VIII/vWF concentrates in all types of vWD.

The treatment with substitutes containing von Willebrand factor in vWD patients, who possess alloantibodies against vWF is contraindicated, because of potential anaphylactic reactions by complement activation (Mannucci et al. 1987, Bergamaschini et al. 1995). Removal of the underlying cause, as for example valve replacement in aortic stenosis, is essential to a resolution of the syndrome for the treatment of acquired vWD (Pareti et al. 2000, Vincentelli et al. 2003).

The aim of this thesis was to analyze the von Willebrand factor (vWF) multimers in their native state and to present Blue Native Electrophoresis (BNE) as a new method of performing vWF multimer analysis, which has not been done before. Another goal was to compare BNE to the commonly used SDS-agarose gel electrophoresis. A previous paper to which this author contributed (Hohenstein et al. 2011) presented the development and advancement of blue native agarose gel electrophoresis for vWF multimer analysis. The method described is a 2-D blue native/SDS gel electrophoresis combining a first-dimensional separation of the multimeric vWF protein in its native state with a denaturing second dimension. “The method aims to identify multiprotein complexes at higher

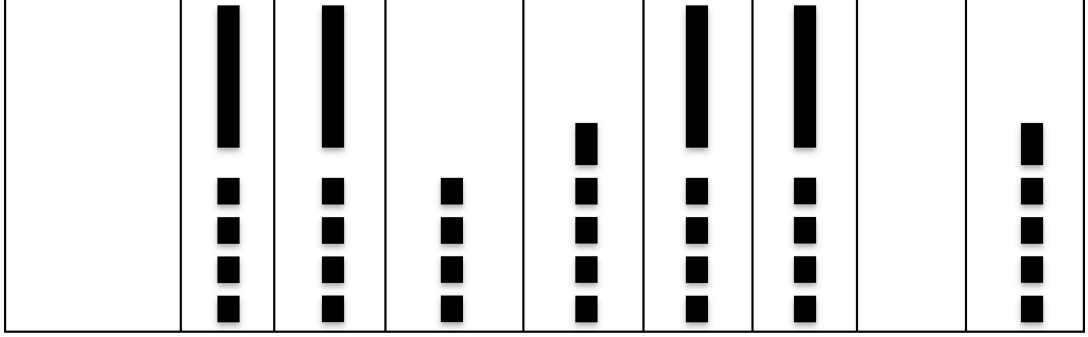
resolution with respect to their subunit composition and has been optimized for utilization in clinical evaluation and classification of patients with vWD ” (Hohenstein et al. 2011, p. 1685).

Tab 1: Expected laboratory values in vWD

vWF:Ag (Von Willebrand factor antigen): It is an immunologic assay which measures quantitatively the vWF antigen but which does not imply functional ability. **vWF:RCo** (Von Willebrand factor ristocetin cofactor activity): This assay measures the ristocetin cofactor activity by quantifying platelet agglutination after addition of ristocetin and vWF. **F VIII** (Factor VIII): It is a plasma-clotting test based on PTT assay using a FVIII-deficient substrate and measures the FVIII activity. **RIPA** (Ristocetin-Induced Platelet Aggregation): This assay measures the ability of the patient's vWF to bind to platelets in the presence of various concentrations of ristocetin. **PFA-100® CT** (platelet function analyzer closure time): It can help in the evaluation of platelet disorders and platelet function. The PFA-100® CT is highly dependent on the vWF, which binds to the platelet membrane glycoprotein receptors. The CT has a higher sensitivity for vWD, compared to the bleeding time. It is abnormal in some acquired and congenital platelet function defects, but it is not prolonged by coagulation factor deficiencies (Hayward et al. 2006). **BT** (bleeding time): It is a coagulation test which assesses platelet function. Bleeding time is affected (prolonged) by platelet dysfunction, certain vascular disorders and von Willebrand Disease, but not by an imbalance of secondary hemostasis (retrenchment of coagulation factors), as in hemophilia. **Pl-Count** (Platelet count): Platelet counts can be done manually with a commercial diluting system, hemocytometer and a microscope or with an automated analyzer by flow cytometry based on principles of light scattering. **vWF multimers** (Von Willebrand factor multimers): vWF multimer analysis is a qualitative assay that depicts the variable concentrations of vWF multimers of different sizes by using SDS-agarose electrophoresis followed by western blot and visualizing them via a specific vWF antibody. **N** (normal): **N*** (normal but with reduced satellite bands). **Von Willebrand factor collagen-binding activity:** This is an ELISA assay which measures the ability of vWF to bind to collagen. This assay has not been established for the evaluation of vWD and is therefore not shown in the table. The sensitivity for discrimination among vWD types depends on the source of collagen (Neugebauer et al. 2002).

Table adapted from National Heart, Lung and Blood Institute. The diagnosis, evaluation, and management of von Willebrand disease. Bethesda, Md.: National Institutes of Health; December 2007:36. NIH publication no. 08-5832. http://www.nhlbi.nih.gov/guidelines/vwd. Accessed April 20, 2012.

	Normal	Type 1	Type 2A	Type 2B	Type 2M	Type 2N	Type 3	PLT-vWD
vWF:Ag	N	↓/↓↓	↓	↓	↓	N/↓	absent	↓
vWF:Rco	N	↓/↓↓	↓↓/↓↓↓	↓↓	↓↓	N/↓	absent	↓↓
FVIII	N	N/ ↓	N/ ↓	N/ ↓	N/ ↓	↓↓	1-9 IU/dl	N/↓
RIPA	N	often N	↓	often N	↓	N	absent	often N
PFA-100® CT	N	N/↑	↑	↑	↑	N	↑↑↑	↑
BT	N	N/↑	↑	↑	↑	N	↑↑↑	↑
Pl-Count	N	N	N	↓/N	N	N	N	↓
vWF multimers	N	N	abnormal	abnormal	N*	N*	absent	abnormal



2 MATERIALS AND METHODS

2.1 Blue native electrophoresis

BNE, originally described by Schägger and von Jagow 1991, was used to isolate the five membrane multiprotein complexes of the oxidative phosphorylation system from bovine mitochondria. It has been applied with great success to the analysis of mitochondrial protein complexes and signal transduction of receptors (Schägger and von Jagow 1991). This technique can be also a very helpful tool to test the purity and homogeneity of chromatographic column eluates, for the detection of protein subcomplexes and for analysis of possible protein-protein interactions of two proteins found in the same fraction (Schägger et al. 1994).

In recent years, this technique has attracted the interest of researchers focusing on functional proteomics, since it enables the separation of proteins under non-denaturing conditions, the study of protein-protein interactions (Krause 2006), and the separation and analysis of highly hydrophobic proteins (Perales et al. 2005). BNE is not restricted to membrane proteins and is also applicable to water-soluble proteins and complexes.

BNE is a charge shift method, in which the electrophoretic mobility of a protein is determined according to its molecular mass, shape and intrinsic charge. In this variation of gel electrophoresis, Serva Blue G (also known as CBB) is used as a charging agent. The dye binds to the surface of the protein and shifts its isoelectric point to a more negative value, so that all proteins, even basic ones, migrate to the anode, regardless of their original isoelectric points, without denaturing them (Schägger 2001). There are neutral and basic water-soluble proteins which do not bind dye. These proteins do not migrate into the gel and are therefore lost. The method is called native, as most separated protein complexes retain enzymatic functions, and blue native, since electrophoretic separation relies on binding of the blue charging agent.

Unlike BNE, SDS electrophoresis uses SDS as the anionic detergent, which denatures secondary and non-disulfide-linked tertiary structures and applies a negative charge to each protein in proportion to its mass. The quaternary structure cannot be investigated with this method. SDS is a strong detergent that causes dissociation of protein complexes and also complete denaturation, whereas Serva Blue G preserves the structure of multiprotein complexes (MPCs) without dissociating them.

The method described in this thesis is a 2-D Blue Native/SDS AGE (Agarose Gel Electrophoresis), combining a first-dimensional separation of the multimeric vWF protein in its native state, followed by a denaturing separation in the second dimension. The second dimension can determine the subunit composition, or rather, the non-dissociated complexes of native multiprotein complexes from the first dimension.

With the introduction of proteomics, the analysis of MPCs has become more important (Gavin et al. 2002). Identification and characterization of MPCs requires analysis under native conditions. One technique to separate protein complexes under native conditions, based on their isoelectric point, is Native Isoelectric Focusing. This method has limited applicability, since many proteins are insoluble at or next to the isoelectric point (Camacho-Carvajal et al. 2004). Other methods, such as gel filtration or sucrose-gradient ultracentrifugation for the native separation of MCPS, have resolution limitations (Schamel and Reth 2000, Schägger and von Jagow 1991). BN-Electrophoresis is an adequate technique for analyzing multiprotein complexes. Membrane surface areas become more hydrophilic by binding the dye, which gives membrane proteins a water-soluble character. This has the advantage that no detergent is required in the BN gels once Coomassie dye has occupied protein surfaces. The risk of denaturing a detergent-sensitive protein is therefore minimized by using BNE (Wittig et al. 2006).

For optimal resolution of a protein of interest it is important to select the right pH for the buffer system. The pH must be within a range in which the protein under investigation is stable. Moreover, the chosen pH for the buffer system must grant sufficient charge to the protein so as to allow adequate migration through the gel in the electric field.

A native protein can have either a positive or negative charge in the agarose, depending on its isoelectric point and the pH of the buffer system used to perform the electrophoresis. For example, proteins that possess a pI lower than the pH of the buffer system carry a negative charge and migrate toward the anode, whereas proteins with a pI higher than the buffer pH carry a positive charge and migrate toward the cathode (Kim et al. 1999).

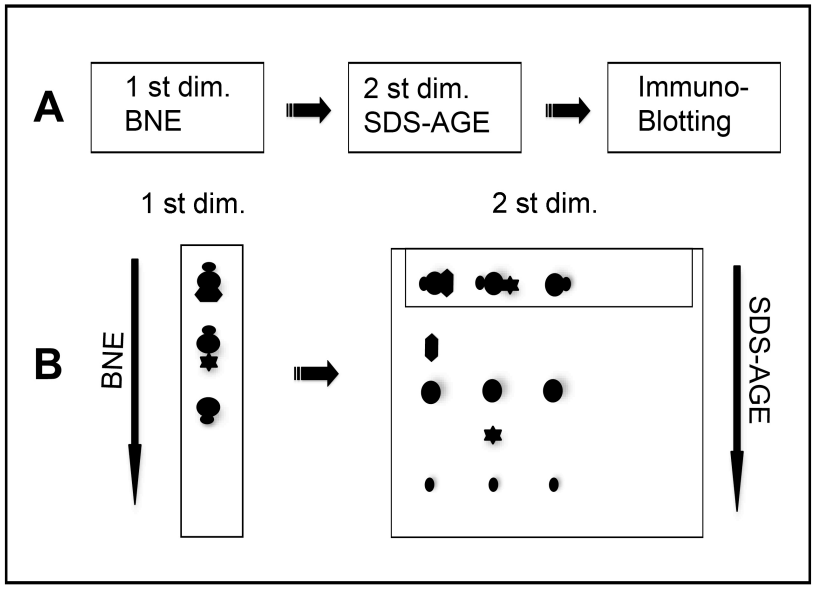


Fig. 1: A, Flow diagram of 2D BN/SDS-AGE. B, Principle of 2D BN/SDS-AGE.

A: The method described in the flow diagram is a 2-D Blue Native/SDS gel electrophoresis combining a first-dimensional separation of a multimeric protein in its native state followed by a denaturing separation in the second dimension. **B:** The diagram shows analysis of protein complexes and corresponding protein subunits after two-dimensional separation. The first dimension (1 st dim.) is a BNE, whereas the second dimension (2 st dim.) is a SDS-AGE. Proteins that are components of the same multiprotein complex (MCP), will be found in one vertical line in the second dimension. Several spots of the same protein in a horizontal line are indicative of the protein being present in several distinct MPCs. Geometrical forms represent single protein subunits which are assembled into a complex.

2.1.1 1-D BN agarose electrophoresis

BNE was performed using a modification of the method described by Schägger and von Jagow (1991) by using agarose as a gel matrix. Commonly, all native electrophoresis systems use acrylamide gradient gels for protein separation. Agarose is an appropriate matrix for the analysis of complexes larger than 1 MDa. The final composition of the low-resolving gel was a 1% wt/vol SeaKem HGT agarose gel, while the percentage of the high-resolving gel was 2% wt/vol. Low-resolution gels (LRG-1%) can demonstrate the change in multimer distribution of the larger multimers (top of the gel), while high-resolution gels (HRG 2%) can separate each multimer into several bands in the so-called triplet structure, which is more distinctive. The buffer system (gel buffer, anode buffer and cathode buffer), at a pH of 8, showed the optimal resolution of the multimers. The ionic concentration in the buffer used in the resolving gel and in the upper (cathode) and lower (anode) electrode chambers differed from each other.

3 RESULTS

The high-sensitivity fluorescence detection of the native Cyanine 5-labeled vWF multimers shows a protein pattern similar to the commonly used SDS-agarose gel electrophoresis. The native immunoblot demonstrates subunit composition patterns, which are repeated regularly in multimers of increasing molecular mass, yielding a series of bands (Hohenstein et al. 2011; figure 1).

The major difference between these methods is the lack of satellite bands in the high-resolution (2%) native gel, which are indispensable for classification of the vWD types (see Table 1). Some of these bands may be missing or may migrate with an aberrant mobility in vWD variants (Batlle et al. 1986; Gralnick et al. 1986; Kinoshita et al. 1984; Mannucci et al. 1986), suggesting that the complex multimeric structure of plasma vWF may have pathophysiological significance. Indeed, the satellite bands are present in patients not suffering from vWD, reduced in vWD type 2M (Budde 2008; Gadisseur et al. 2009), and absent in a TTP plasma (Schneppenheim et al. 2003).

To analyze this phenomenon further, a second dimension was performed under denaturing conditions. SDS should denature the native protein complexes and resolve them into subunits. The first dimension was performed in a low-resolution (1%) native agarose gel in contrast to the second dimension, where a high-resolution (2%) SDS agarose gel was used. As expected, a pattern was obtained in which each protein band from the first dimension dissociated into three subbands (Hohenstein et al. 2011; figure 2). These subbands confirm the characteristic triplet structure, which consists of a more prominent intermediate band and two satellite bands, one migrating faster, and the other more slowly (Aihara et al 1986; Ruggeri and Zimmerman 1981; Siedlecki et al. 1996).

The absence of the characteristic triplet subband structure in platelet vWF shown by SDS multimer analysis (Dent et al. 1991; Ott et al. 2010), could not be confirmed properly with the use of 2-D blue native/SDS-agarose electrophoresis. The second dimension of normal platelet vWF even showed a triplet structure (Hohenstein et al. 2011; figure 4). This observation has never been made previously without using BNE technique.

Furthermore, an additional, unknown, slowly migrating band not present in plasmatic vWF was identified by the 2-D native/SDS immunoblot (Hohenstein et al. 2011; figure 4). This additional band belongs to the triplet structure and confirms the supposition that the platelet vWF consists of a quadruplet structure. The possibility that the results of the 2-D

multimeric organization of the platelet vWF are due to proteolytic modification is unlikely, since the platelet vWF was collected in the presence of protease inhibitors.

The appearance of the triplet structure of vWF is interpreted as a proteolytic product of vWF multimers from the metalloprotease ADAMTS13 (Fernandez et al. 1982, Furlan et al. 1993). The role of ADAMTS13 in the degradation of vWF has been well established (Levy et al. 2001; Zheng et al. 2001). The missing triplet structure in platelet vWF, shown by the SDS-agarose electrophoresis (Dent et al. 1991; Ott et al. 2010), reflects the absence of proteolytic degradation. Proteolytic cleavage of plasma vWF subunits occurs after the release from cellular sites (Dent et al. 1991), whereas the analyzed platelet vWF was recovered from lysed platelets and was therefore protected from proteolysis.

Furthermore, a study published by Schneppenheim et al. (2003) supports the hypothesis that satellite bands are a product of proteolysis. The authors report the absence of the satellite bands in a TTP plasma, whereas they appear after the addition of ADAMTS13. The triplet structure of platelet vWF has not been identified previously through the conventional SDS-agarose-electrophoresis multimer analysis. In contrast, analysis of the platelet vWF by 2-D blue native/SDS-agarose electrophoresis illustrates clearly defined satellite bands. This raises the question of whether the appearance of the triplet structure of vWF multimers results from proteolytic degradation or is an artifact caused by SDS. Owing to the fact that we applied complete protease inhibitor cocktail tablets for the extraction of the platelet vWF, and to the claim that the glycoprotein is stored in the alpha granules and is therefore protected from proteolytic degradation (Dent et al. 1991), the appearance of the triplet structure is not based on the proteolysis assumption.

The finding that the satellite bands of the platelet vWF are not resolved by the high-resolution native electrophoresis but through the second SDS dimension indicates that SDS may play an important role in the formation of the triplet structure because of its property of unfolding the shape of the vWF protein. In addition, a reason why the commonly applied SDS-agarose electrophoresis cannot resolve the platelet vWF satellite bands remains elusive.

Until now, whether and/or how the satellite bands are represented in vWF multimers has not been clarified. Nevertheless, they have pathophysiological significance and are useful for the classification of the vWD. Furthermore, the two-dimensional pattern of platelet vWF shows an additional unidentified band, which suggests that the multimeric composition of vWF is more complex than has been previously thought. Evaluating

possible explanations for the presence of an unidentified band is hampered by our inability to sequence it, due to its low protein concentration in the second-dimension gel.

SDS-agarose electrophoresis is a valuable tool for rapid screening for vWD, which makes it diagnostically and clinically relevant. In contrast, the blue native method is more time-consuming due to the second dimension. Thanks to the higher resolving power, this method is a helpful tool for classifying ambiguous subtypes.

The 2D- Blue Native/SDS gel electrophoresis has the additional advantage of depicting the triplet structure of platelet vWF multimers, which enables us to compare the subunit structures from platelet and plasmatic vWF. This may help to find out whether structural abnormalities concern the vWF molecule in the platelet itself or whether they are due to the physiological processing (cleavage of the constituent subunit, for example) of vWF after secretion from the cell into circulation. Thereby, the standard classification of vWD, which is based mainly on multimeric analysis of plasmatic vWF, could be extended by additional analysis of the platelet vWF.

Furthermore, native electrophoresis allows additional analysis of protein-protein interactions. With this newly described method we seek to take a step toward a more effective classification of the subtypes of vWD in human plasma and platelet lysates. We hope that further experiments can shed light on the mechanism of the formation of satellite bands in vWF multimers.

4 SUMMARY

The analysis of vWF multimers by electrophoretic separation is an important laboratory tool for distinguishing the subtypes of vWD. Blue native electrophoresis is a new method for performing vWF multimer analysis and it is compared in this thesis to the commonly used SDS-agarose gel electrophoresis.

The method described here is a 2-D blue native/SDS gel electrophoresis combining a first-dimensional separation of the multimeric vWF protein in its native state followed by a denaturing separation in the second dimension.

The major difference between this method and the commonly used SDS-agarose gel electrophoresis is the lack of satellite bands in the high-resolution native gel. In the second dimension a pattern was obtained where each protein subunit from the first dimension dissociates into three distinct subbands. These bands confirm the triplet structure, which consists of an intermediate band and two satellite bands. Our method separates the triplet structure into a higher resolution than the commonly used SDS-agarose gel electrophoresis does. This helps considerably in the classification of ambiguous vWD-subtypes. The 2D-blue native/SDS gel electrophoresis has the additional advantage of being able to resolve the triplet structure of platelet vWF multimers, which has not been previously identified through conventional SDS-agarose electrophoresis multimer analysis. This potential enables us to compare the triplet structures from platelet and plasmatic vWF and may help to find out whether structural abnormalities concern the vWF molecule in the platelet itself, or are due to the physiological processing of vWF shedded into circulation.

Due to its resolution and sensitivity, this native separation technique offers a promising tool for the classification of von vWD-subtypes.

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6 PUBLICATION

Hohenstein K, Griesmacher A, Weigel G, Golderer G, Ott HW (2011):

Native multimer analysis of plasma and platelet von Willebrand factor compared to denaturing separation: Implication for the interpretation of satellite bands.

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Research Article

Native multimer analysis of plasma and platelet von Willebrand factor compared to denaturing separation: Implication for the interpretation of satellite bands

Blue native electrophoresis (BNE) was applied to analyze the von Willebrand factor (vWF) multimers in their native state and to present a methodology to perform blue native electrophoresis on human plasma proteins, which has not been done before. The major difference between this method and the commonly used SDS-agarose gel electrophoresis is the lack of satellite bands in the high-resolution native gel. To further analyze this phenomenon, a second dimension was performed under denaturing conditions. Thereby, we obtained a pattern in which each protein sub-unit from the first dimension dissociates into three distinct sub-bands. These bands confirm the triplet structure, which consists of an intermediate band and two satellite bands. By introducing the second dimension, our novel method separates the triplet structure into a higher resolution than the commonly used SDS-agarose gel electrophoresis does. This helps considerably in the classification of ambiguous von Willebrand's disease subtypes. In addition, our method has the additional advantage of being able to resolve the triplet structure of platelet vWF multimers, which has not been identified previously through conventional SDS-agarose electrophoresis multimer analysis. This potential enables us to compare the triplet structure from platelet and plasmatic vWF, and may help to find out whether structural abnormalities concern the vWF molecule in the platelet itself, or whether they are due to the physiological processing of vWF shed into circulation. Owing to its resolution and sensitivity, this native separation technique offers a promising tool for the analysis and detection of von Willebrand disorder, and for the classification of von Willebrand's disease subtypes.

Keywords:
Blue native agarose electrophoresis / von Willebrand disease / von Willebrand factor / von Willebrand multimer analysis DOI 10.1002/elps.201000662

1 Introduction

von Willebrand factor (vWF) is a large, adhesive, multimeric glycoprotein, which supports platelet adhesion and aggregation to the sub-endothelium in primary hemostasis [1], and stabilizes factor VIII during secondary hemostasis by acting as a carrier and protector for factor VIII [2]. The protein circulates in human plasma at concentrations of between 8 and 25 μg/mL [3] as a series of heterogeneous multimers ranging in size from about 500 kDa to over 20 000 kDa [4]. vWF is synthesized in vascular endothelial cells [5] and megakaryocytes [6] and is stored in Weible Palade bodies of endothelial cells as well as in the α granules of platelets [7]. Qualitative and/or quantitative abnormalities of vWF can be identified in patients who suffer from von Willebrand's disease (vWD), a common inherited bleeding disorder with an estimated prevalence of up to 1% [8–10].

vWD can be classified into three types depending upon whether the individual defect is quantitative (types 1 and 3) and/or qualitative (types 2 and 3) [10]. Type 2 is further divided into several subtypes (2A, 2B, 2M, and 2N). Most cases of vWD are hereditary, but abnormalities in the protein can also be acquired and are involved in a large number of other diseases, including thrombotic thrombocytopenic purpura (TTP), Heyde's syndrome, and hemolytic uremic syndrome [11].

Diagnosis of vWD is based on the clinical history and measurement of the functional properties of the factor VIII/vWF complex, including vWF antigen, ristocetin cofactor activity, and factor VIII coagulant activity. Ristocetin-

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Abbreviations: BNE, blue native electrophoresis; **PBS-T,** PBS-0.05% Tween-20 solution; **TTP,** thrombotic thrombocytopenic purpura; **vWD,** von Willebrand's disease; **vWF,** von Willebrand factor

Colour Online: See the article online to view Fig. 3 in colour.

induced platelet aggregation, vWF collagen-binding test, and vWF multimeric pattern evaluation are utilized for the classification of vWD subtypes [15, 16]. The occurrence of satellite bands contributes currently to sub-class identification (see vWF-type 2M and 2N in Table 1). Typically, the visualization of vWF multimers has been based on SDS-agarose gel electrophoresis [17, 18].

In this article, we present the development and advancement of blue native agarose gel electrophoresis for vWF multimer analysis. The method we describe is a 2-D blue native/SDS gel electrophoresis combining a first-dimensional separation of the multimeric vWF protein in its native state followed by a denaturing separation in the second dimension. The method aims to identify multi-protein complexes at higher resolution with respect to their sub-unit composition and has been optimized for utilization in clinical evaluation and classification of patients with vWD. Blue native electrophoresis (BNE), originally described by Schägger and von Jagow [19], was used to isolate the five membrane multiprotein complexes of the oxidative phosphorylation system from bovine mitochondria. In the recent years, this technique has attracted the interest of researchers focusing on functional proteomics, since it enables the separation of proteins under non-denaturing conditions, the study of protein–protein interactions [20], and the separation and analysis of highly hydrophobic proteins [21]. In this variation of gel electrophoresis, the anionic dye Serva Blue G (also known as CBB) is used as charging agent that binds to the surface of proteins. It shifts the isoelectric point of the proteins to more negative values so that all proteins, even basic ones, migrate to the anode according to their molecular mass, intrinsic charge, and shape regardless of their original isoelectric points without denaturing them [22]. Unlike native electrophoresis, SDS electrophoresis uses SDS as the anionic detergent, which denatures secondary and non-disulfide-linked tertiary structures and applies a negative charge to each protein in proportion to its mass. The quaternary structure cannot be investigated with this method. In the present study, we explore whether the application of BNE is also applicable to vWF multimeric analysis. In addition, we compare it to the commonly used SDS-agarose gel electrophoresis [18], and seek to improve diagnosis of vWD in so doing.

2 Materials and methods

2.1 Reagents

SeaKem HGT-P (High-Gelling Temperature, ultra Pure) agarose was purchased from Lonza (Verviers, Belgium); urea, EDTA disodium salt, SDS, polyoxyethylene sorbitan monolaurate (Tween-20), bovine serum albumin, sodium chloride, potassium chloride, magnesium chloride, sodium bicarbonate, hydrochloric acid, and bromophenol blue were purchased from Sigma (St Louis, MO, USA); methyl alcohol, TRIS, glycine, and Bis-TRIS from Merck (Darmstadt, Germany); SERVA Blue G (also known as CBB G 250) from Serva; complete protease inhibitor cocktail tablets from Roche and PBS tablets from Medicago. All chemicals were of analytical grade.

2.2 Sample information

Correct identification of analyzed samples was confirmed by participation in the ECAT vWF ring trial and by testing in a reference laboratory (Prof. Spannagl, Munich, Germany).

As far as the reproducibility of gel profiles is concerned, plasmatic and platelet vWF samples have been analyzed repeatedly.

vWD-type	Sample identification	1-D gel[a)]	2-D gel
vWD-type NP	ECAT/10.13[b)]	$n = 10$	$n = 5$
vWD-type 2B	ECAT/99[b)]	$n = 2$	$n = 2$
vWD-type 2M	External validation[c)]	$n = 2$	$n = 2$
Platelet-vWF	Platelet concentrate	$n = 2$	$n = 2$
Platelet-vWF	Platelet-rich plasma preparation of normal plasma	$n = 2$	$n = 2$

a) For the 1-D gel each experiment was done for the 1 and 2% agarose gel; NP: normal plasma; n = number of experiments.
b) ECAT Foundation.
c) External validation by Professor Spannagl's reference laboratory, Munich

2.3 Platelet-poor plasma preparation

Plasma was prepared from freshly drawn whole blood (one part 3.8% sodium citrate, nine parts whole blood) by centrifugation at room temperature at $2100 \times g$ for 15 min. Platelet-poor plasma was obtained by centrifugation at $10\,000 \times g$ for 5 min. Platelet-poor plasma was diluted in sample buffer (50 mM Bis-TRIS, 0.5 mM EDTA-Na_2, 20% Glycerol, 0.1% SERVA Blue G), according to the vWF-Ag (%) content (measured immunologically by a CE-labeled vWF Ag test kit – -Siemens Healthcare Diagnostics). The dilution factor (DF) was calculated according to the formula:

$$DF = \frac{\left(\frac{vWF-Ag(\%)}{100}\right)}{0.05}$$

2.4 Platelet-rich plasma preparation

Plasma was prepared from freshly drawn whole blood (one part 3.8% sodium citrate, nine parts whole blood) by centrifugation at room temperature at $500 \times g$ for 5 min.

Table 1. Characteristics of von Willebrand disease types

Type	Bleeding time	Factor VIII:C	vWF:AG	vWF:CB	vWF Multimers
1	Normal/prolonged	Normal/reduced	Reduced	Reduced	Normal
2A	Prolonged	Normal/reduced	Normal/reduced	Reduced	Absence of large and middle vWF
2B	Prolonged	Normal/reduced	Normal/reduced	Reduced	Absence of large vWF
2M	Normal/prolonged	Normal/reduced	Normal/reduced	Normal/reduced	reduced satellite bands[a)]
2N	Normal	Reduced	Normal/reduced	Normal/reduced	reduced satellite bands[b)]
3	Markedly prolonged	Reduced	Below limit of detection	Below limit of detection	Below limit of detection

Factor VIII:C, factor VIII activity; vWF:AG, vWF antigen; vWF:CB, vWF collagen-binding test.
a) [12, 13].
b) [14].

2.5 Extraction of platelet vWF

Before platelets were pelleted at $300 \times g$ for 10 min at room temperature, platelet-rich plasma had been centrifuged at $100 \times g$ for 5 min in order to remove contaminating blood cells and plasmatic vWF. The platelet pellet was resuspended gently in 10 mL washing buffer (PBS, pH 7.4, supplemented with complete protease inhibitor cocktail) and centrifuged at $1000 \times g$ for 10 min. This washing procedure was repeated twice. Before the platelet pellet was lysed in A. bidest, the vWF AG (%) was measured immunologically (Behring Coagulation System analyzer – Siemens) in the supernatant of the final wash step to ensure that the platelet suspension was free of plasmatic vWF. Platelets were lysed by repeated freezing–thawing and the lysate was centrifuged at $1300 \times g$ at 4°C for 10 min. The supernatant was collected, aliquoted, and stored at −80°C for up to 1 month.

2.6 Electrophoresis

Vertical electrophoresis was carried out using a SE-600 Ruby Standard Dual-Cooled Gel Electrophoresis Unit (GE Healthcare Life Sciences, Vienna, Austria).

2.7 1-D BNE

BNE was performed using a modification of the method described by Schagger and von Jagow [19]. Agarose was used as a gel matrix instead of polyacrylamide. Agarose is an appropriate matrix for the analysis of complexes larger than 1 MDa. Vertical BNE was carried out at a constant voltage of 50 V overnight (approximately 12 h). The gel chamber was cooled to 10°C with a circulating water bath. The final compositions of the two resolving gels were 1% wt/vol (low-resolving gel) and 2% wt/vol (high-resolving gel) SeaKem HGT agarose.

2.8 Buffers for 1-D

Gel buffer: 50 mM Bis-Tris, pH 8

Anode buffer: 25 mM Bis-Tris, pH 8; cathode buffer: 50 mM Tricine, 15 mM Bis-Tris, 0.02% Serva Blue G, pH 8.

2.9 Casting of the gel

Gel dimensions were 16×16 cm. The spacers used were 1.5 mm thick.

2.10 2-D SDS-agarose electrophoresis

The protein complexes resolved in the first dimension were denatured by incubating the 1-D agarose gel in dissociating solution (70 mM Tris, 4 mM EDTA disodium salt, 9 M urea, 1% v/v SDS, pH 6.8) for 1 h at room temperature with gentle agitation. An individual lane was cut out of the 1-D gel with a razor blade and excess dissociating solution was drained off using filter paper. The strip was then placed horizontally onto a glass plate at the lower edge. Spacers were positioned, covered with the second glass plate, and the 2-D agarose gel was cast on the 1-D strip, which had been stuck between the assembled glass plates at their lower edges. Electrophoresis was performed at a constant voltage of 100 V for approximately 8 h. The gel chamber was cooled to 10°C with a circulating water bath.

2.11 Buffers for 2-D

Gel buffer: 198 mM Tris, 93 mM glycine, 4 mM EDTA, 0.4% SDS; pH not adjusted; electrophoresis buffer: 99 mM Tris, 146 mM glycine, 0.1% SDS; pH not adjusted.

2.12 Western blotting

Proteins were electrotransferred onto a PVDF 0.45 μm Hybond-LFP low-fluorescent membrane (GE Healthcare Life Sciences) for 1.5 h at 130 V constant voltage using a Trans-Blot cell with plate electrodes (Hoefer TE 62 Cooled Transfer Electrophoresis Unit, GE Healthcare Life Sciences) in a buffer containing 25 mM Tris, 200 mM glycine, 20% v/v methanol and 0.1% v/v SDS. The blot chamber was cooled to 10°C with a circulating water bath.

2.13 Blocking and washing strategy

After transfer, the blot membrane was saturated with 2% ECL advanced blocking agent (GE Healthcare Life Sciences) diluted in PBS-0.05% Tween-20 solution (PBS-T), pH 7.4, with agitation for 1 h at room temperature. The membrane was rinsed three times in PBS-T.

2.14 Immunolocalization and densitometric analysis of vWF multimers

Immunolocalization and densitometric analysis of vWF multimers were carried out as described in [18]. In brief, the blocked and washed membrane was incubated with the first antibody (rabbit anti-human vWF DAKO, diluted 1:3000 in PBS-T) for 1 h at room temperature with mild agitation, followed by three washing steps for 5 min. Later, the membrane was incubated with the second antibody (Cy5-labeled ECL Plex goat-anti rabbit IgG; GE Healthcare Life Sciences) for 1 h at room temperature under gentle agitation. The second antibody was reconstituted in ultrapure water to a concentration of 1 μg/μL and was then diluted 1:300 in PBS-T. The detection of the Cy5-labeled vWF multimers on the PVDF membrane was carried out by using a Typhoon 9410 (GE Healthcare Life Sciences) fluorescent laser scanner.

3 Results

3.1 Comparison of multimeric structure of Wilfactine® (purified vWF), normal plasma VWF, vWD-type 2M, and vWD-type 2B plasma samples with SDS-and blue native-agarose gel electrophoresis

The high-sensitivity fluorescence detection of the native Cyanine 5-labeled vWF multimers (see lanes 9–16 in Fig. 1B) shows a similar protein pattern to the commonly used SDS-agarose gel electrophoresis (see lanes 1–8 in Fig. 1A).

The characteristic triplet sub-band structure of plasmatic vWF, as the immunoblot from the high-resolution SDS agarose gel shows (lane 5, lane 6, and lane 8) and the reduced triplet structure of vWD-type 2M (the slower satellite band is missing; see lane 7) was identified neither in the low-resolution gel nor in the high-resolution gel by the native separation of vWF.

In both methods the plasmatic vWF multimeric pattern of vWF-type 2B shows clear differences when compared to the vWF of normal plasma (see Fig. 1A/B). The multimeric structure illustrates a loss of the high-molecular-weight multimers in type 2B (see lanes 4, 8, 12, and 16 in Fig. 1A/B), which assigns it to this sub-type.

In our experiments Wilfactine® (see lanes 1/5/9/13 in Fig. 1A/B), a purified vWF-product was used as a control.

3.2 Comparison of 2-D plasmatic vWF multimeric structure of Wilfactine® (purified vWF), normal plasma VWF, vWD-type 2M, and vWD-type 2B by blue native and SDS agarose gel electrophoresis

The major difference between the blue native and SDS-agarose gel electrophoresis is the lack of the satellite bands in the high-resolution native gel. To analyze this phenomenon further, a second dimension was performed under denaturing conditions. SDS should denature the native protein complexes and resolve them into sub-units.

As expected, we obtained a pattern in which each protein band from the first dimension dissociates into three sub-bands (see numbered bars in Fig. 2A, B and D). These sub-bands confirm the triplet structure, which consists of a more prominent intermediate band, and two satellite bands, one migrating faster, the other more slowly.

Figure 2C illustrates the identification of vWD-type 2M obtained by our combination of 2-D blue native and SDS-agarose electrophoresis. The vWF-type 2M is not easy to

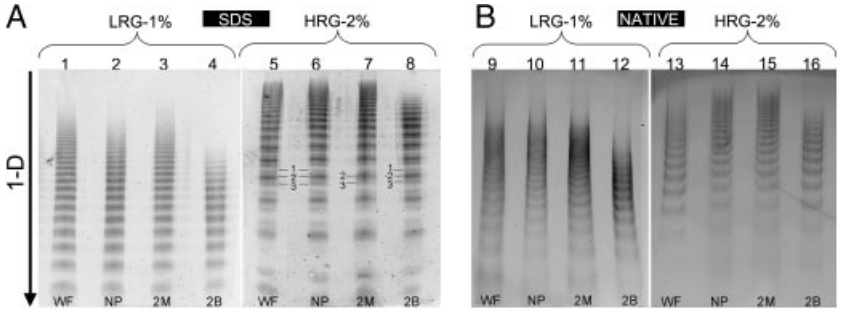

Figure 1. Comparison of vWF multimer analyses by SDS and blue native-agarose gel electrophoresis of Wilfactine® (purified vWF) (WF), normal plasma vWF (NP), vWD-type 2 M (2 M) and vWD-type 2B (2B). (A) Fluorescence-immunoblot of SDS low-resolution agarose gel (LRG: 1%) and highresolution (HRG 2%) agarose gel. The numbered bars in lane 5, lane 6, and lane 8 indicate the resolved triplet structure: 1 a slower migrating satellite band, 2 an intermediate band, and 3 a faster migrating band. The pattern in lane 7 shows a reduced configuration of the triplet sub-bands: 2 an intermediate band and 3 a faster migrating band. The slower migrating satellite band is missing. (B) Fluorescence-immunoblot of blue native low-resolution agarose gel (LRG: 1%) and high-resolution (HRG: 2%) agarose gel.

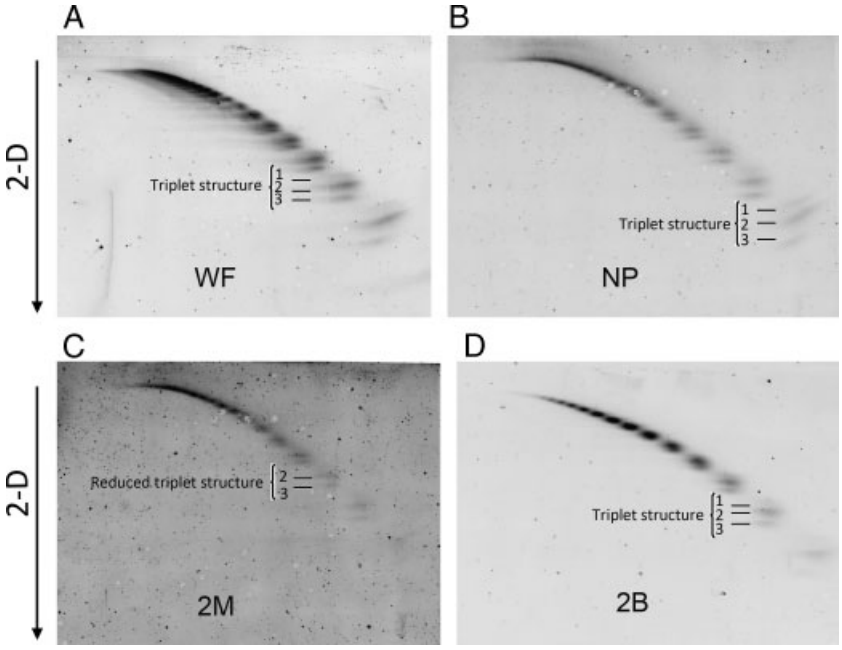

Figure 2. Comparison of 2-D blue native/SDS-agarose electrophoresis pattern of Wilfactine® (purified vWF, as a control) (A), normal plasma vWF (B), vWD-type 2M (C), vWD-type 2B (D). The first dimension was performed in a low-resolution (1%) native agarose gel in contrast to the second dimension where a high-resolution (2%) SDS agarose gel was used. Arrow indicates the direction of the 2-D. (A) Fluorescence-immunoblot of Wilfactine® (WF): The numbered bars in the bracket indicate the resolved triplet structure: 1 a slower migrating satellite band, 2 an intermediate band, and 3 a faster migrating band. (B) Fluorescence-immunoblot of normal plasma vWF (NP): The pattern shows a normal configuration of the triplet sub-bands (see numbered bars in brackets). (C) Fluorescenceimmunoblot of plasmatic vWF of VWD-type 2M (2M): The pattern shows a reduced configuration of the triplet sub-bands: 2 an intermediate band and 3 a faster migrating band. Note that the slower migrating satellite band is missing. (D) Fluorescence-immunoblot of plasmatic vWF of VWD-type 2B (2B): The pattern shows a normal configuration of the triplet sub-bands (see numbered bars in bracket).

determine by multimeric analysis. Normal antigen levels characterize this type. A qualitative deficit in vWF is seen only in a reduced ristocetin cofactor activity and not in an increase of high-molecular-weight multimers [23].

Reduced satellite bands in the triplet structure (see numbered bars in brackets in Fig. 2C; the slower satellite band is missing) and a normal multimer pattern [12, 13] classify it as the vWD sub-type 2M.

3.3 Comparison of the multimeric structure of normal vWF in platelets and plasma by BNE

The native multimeric structure of plasmatic and platelet vWF was analyzed in high-resolution (2%) agarose gel. Characteristic differences were found and are presented in Fig. 3A. The native multimeric structure of platelet vWF in the first dimension (see lane 2 Fig. 3A) illustrates additional multimers of higher molecular weight. This phenomenon has also been demonstrated in previous studies [18, 24] where platelet vWF was separated by SDS-agarose electrophoresis. The presence of the larger multimers in platelet vWF is clearly shown by the densitometric quantification of the native multimeric patterns in Fig. 3B.

Our studies do not rule out the possibility that the multimeric differences between platelet and plasma vWF are due to intracellular processing prior to release into circulation. Unlike the platelet vWF, the plasmatic glycoprotein has already undergone intracellular processing prior to release from endothelial cells, whereas the platelet vWF is recovered directly from lysed platelets. However, the presence of the characteristic triplet sub-band structure in plasmatic vWF, and its absence in platelet vWF [18] shown by SDS multimer analysis, could not be affirmed properly due to the 1-D BNE. The triplet sub-band structure in the native high-resolution agarose gel was identified neither in plasmatic vWF nor in platelet-vWF.

3.4 2-D multimeric composition of platelet vWF of a patient not suffering from vWD

In order to verify whether platelet vWF possesses a triplet structure, a 2-D blue native/SDS-agarose electrophoresis was performed. The second dimension of normal platelet vWF shows a triplet structure in Fig. 4. This observation has never previously been made [18, 25] without using BNE technique. Furthermore, an additional unknown slowly migrating band (see encircled area in Fig. 4) not present in plasma was identified by the 2-D native/SDS immunoblot. Viewing the 2-D blot accurately it becomes clear that each additional slowly migrating band belongs to the triplet structure below it. This confirms the supposition that the platelet vWF consists of a quadruplet structure. The possibility that the results of the 2-D

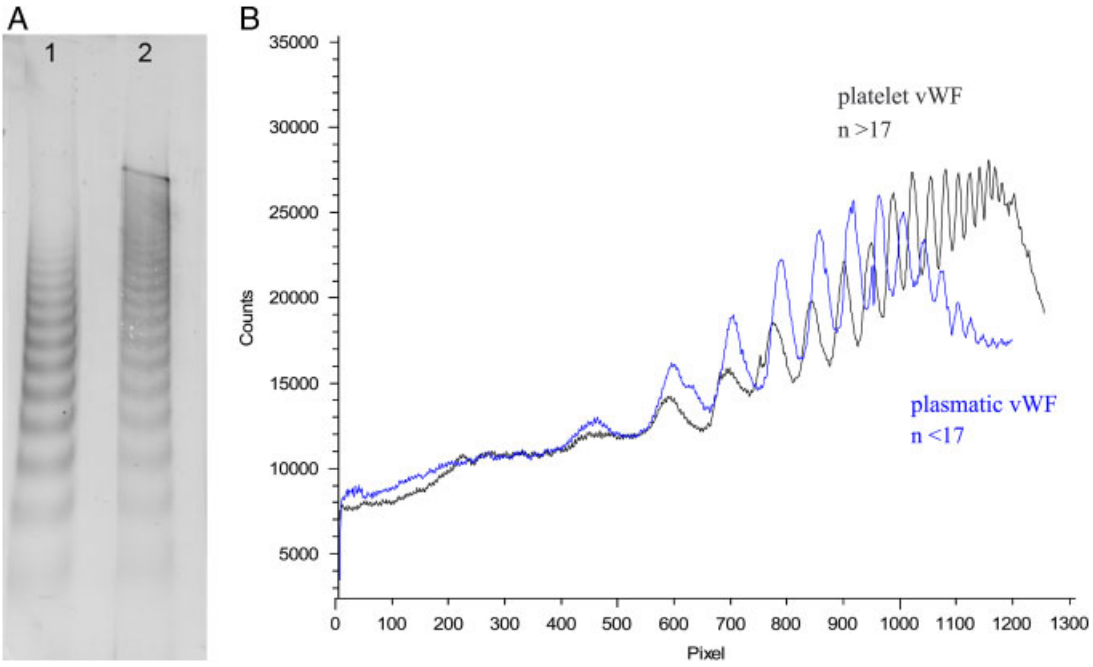

Figure 3. Comparison of multimeric analyses of normal plasmatic and platelet vWF by BNE. (A) 1-D blue native high-resolution agarose gel pattern of plasmatic – (lane1) and platelet vWF (lane2), visualized by high-sensitivity fluorescence detection. (B) Densitometric quantification of two different multimeric patterns of plasmatic vWF (blue line) and platelet vWF (black line) in high-resolution (2%) gel.

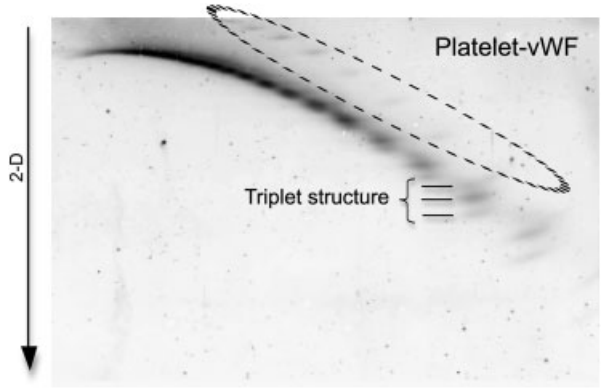

Figure 4. 2-D blue native/SDS-agarose electrophoresis pattern of normal platelet vWF, visualized by high-sensitivity fluorescence detection. Platelet vWF was extracted in the presence of Complete Protease Inhibitor Cocktail. The bracket indicates the triplet structure resolved in the gel. The encircled area shows an additional slowly migrating band, present in platelet vWF. Each triplet structure in combination with its respective slowly migrating band forms a quadruplet structure. The arrow indicates direction of the 2-D.

multimeric organization of the platelet vWF are due to proteolytic modification is unlikely, since the platelet vWF was collected in the presence of protease inhibitors.

4 Discussion

The analysis of vWF multimers by electrophoretic separation and subsequent visualization is an important laboratory tool for distinguishing the subtypes of vWD. Treatment of vWD is based on the identification of the specific subtypes; therefore the adequate classification of the patient's disease plays a key role in therapy.

In this article we present BNE as a new method of performing vWF multimer analysis and compare it to the commonly used SDS-agarose gel electrophoresis. The native immunoblot shows sub-unit composition patterns, which are repeated regularly in multimers of increasing molecular mass, yielding a series of bands with a similar structure. The major difference between these methods is the lack of satellite bands in the high-resolution (2%) native gel (see Fig. 1), which are indispensable for classification of the vWD types (Table 1). This disadvantage can be countered by the use of a second dimension. Using the second dimension lengthens this method considerably. Owing to this second dimension, however, the triplet structure shows a higher resolution than in the commonly used SDS-agarose gel electrophoresis. The triplet structure consists of a more prominent intermediate band, a faster migrating band, and a slower migrating satellite band [26–28]. Some of these bands may be missing or may migrate with an aberrant mobility in vWD variants [29–32], suggesting that the complex multimeric structure of plasma vWF may have pathophysiological significance. Indeed, the satellite bands are present in patients not suffering from vWD, reduced in vWD-type 2M [12, 13], and absent in a TTP plasma [33].

The appearance of the triplet structure of vWF is interpreted as a proteolytic product of vWF multimers from the metalloprotease ADAMTS13 [24, 34]. The role of

ADAMTS13 in the degradation of vWF has been well established [35, 36]. The missing triplet structure in platelet vWF, shown by the SDS-agarose electrophoresis [18, 25], reflects the absence of proteolytic degradation. Proteolytic cleavage of plasma vWF sub-units occurs after the release from cellular sites [25], whereas the analyzed platelet vWF was recovered from lysed platelets and therefore protected from proteolysis. Furthermore, a study published by Schneppenheim et al. [33] supports the hypothesis that satellite bands are a product of proteolysis. The authors report the absence of the satellite bands in a TTP plasma, whereas they appear after the addition of ADAMTS13. The triplet structure of platelet vWF has not been identified previously through the conventional SDS-agarose-electrophoresis multimer analysis. In contrast, analysis of the platelet vWF by 2-D blue native/SDS-agarose electrophoresis illustrates clearly defined satellite bands. This raises the question whether the appearance of the triplet structure of vWF multimers results from proteolytic degradation or whether it is an artifact caused by SDS. Owing to the fact that we applied complete protease inhibitor cocktail tablets for the extraction of the platelet vWF, and the claim that the glycoprotein is stored in the α granules and therefore protected from proteolytic degradation [25], the appearance of the triplet structure is not based on the proteolysis assumption. The finding that the satellite bands of the platelet vWF are not resolved by the high-resolution native electrophoresis but through the second SDS dimension indicates that SDS may play an important role in the formation of the triplet structure because of its property of unfolding the shape of the vWF protein. In addition, the possible reasons why the commonly applied SDS-agarose electrophoresis cannot resolve the platelet vWF satellite bands remains ambiguous. Up to now, whether and or how the satellite bands are represented in vWF multimers has not been clarified. Nevertheless, they have pathophysiological significance and are useful for the classification of the vWD (see Table 1). Furthermore, the two-dimensional pattern of platelet vWF demonstrates an additional unidentified band, which suggests that the multimeric composition of vWF is more complex than has been previously thought. Evaluating possible explanations for the presence of an unidentified band is hampered by our inability to sequence it, due to its low protein concentration in the second-dimension gel.

SDS-agarose electrophoresis is a valuable tool for rapid screening for vWD and makes it diagnostically and clinically relevant. In contrast, the blue native method is more time consuming due to the second dimension. Thanks to the higher resolving power, this method is a helpful tool for classifying ambiguous sub-types. Our method has the additional advantage of depicting the triplet structure of platelet vWF multimers, which enables us to compare the sub-unit structures from platelet and plasmatic vWF. This may help to find out whether structural abnormalities concern the vWF molecule in the platelet itself or whether they are due to the physiological processing (cleavage of the constituent subunit, for example) of vWF after secretion from the cell into circulation. Thereby, the standard classification of vWD, which is based mainly on multimeric analysis of plasmatic vWF, could be extended by additional analysis of the platelet vWF. Furthermore, native electrophoresis allows additional analysis of protein–protein interactions. With this newly described method we seek to make a step toward a more effective classification of the subtypes of vWD in human plasma and platelet lysates. We hope that further experiments can shed light on the mechanism of the formation of satellite bands in vWF multimers.

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