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Plasma von Willebrand factor multimer quantitative analysis by in-gel immunostaining and infrared fluorescent imaging

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ABSTRACT

Introduction: Electrophoretic analysis of plasma von Willebrand factor (VWF) multimer distribution and infrastructure is essential for subtyping von Willebrand disease. To improve the sensitivity, precision and efficiency of this assay, we developed and validated a new in-gel infrared fluorescent VWF multimer imaging method to visualize and quantify VWF multimers directly in the agarose gel, thus eliminating electroblotting or autoradiographic steps.

Materials/Methods: VWF multimer analyses of plasma samples from 34 patients with known von Willebrand disease or acquired von Willebrand syndrome, 9 patients with acquired VWF abnormalities, 26 normal volunteer donors and 49 patient samples referred for von Willebrand factor multimer analysis were performed by both traditional autoradiographic and the new infra-red imaging methods and compared. VWF multimer image data were electronically acquired, archived and analyzed.

Results: The in-gel infrared method has a sensitivity of detecting VWF antigen as low as approximately 1.6 IU/dL, a reliable fluorescent intensity with intra- and inter-day variability (CV) of 5% and 6% respectively, and provides superior imaging resolution and shortened test turnaround time. Using intermediate resolution agarose gel electrophoresis, the infra-red method sensitively detects subtle loss of highest molecular weight von Willebrand factor multimers in plasmas with acquired VWF abnormalities and in commercial normal reference plasmas, and provides evidence of increased proteolysis of ultralarge multimers in some type 2 VWD plasmas.

Conclusions: The in-gel infrared fluorescent VWF multimer imaging method provides a sensitive, reliable, efficient and robust system to improve laboratory testing for von Willebrand disease classification.

Von Willebrand disease (VWD) is an autosomal hereditary disorder due to quantitative or qualitative defects of von Willebrand factor (VWF), and is the most commonly inherited bleeding disorder [1,2], but may also be acquired (acquired von Willebrand syndrome [AVWS]) [1]. Diagnosis of VWD entails a personal and/or a family history of abnormal clinical bleeding, and supportive laboratory test results which include assays of plasma VWF antigen (VWF:Ag), VWF ristocetin cofactor activity (VWF:RCo), and factor VIII activity (FVIII:C), supplemented with plasma VWF multimer analysis and other testing for VWD subtyping [1,3]. VWD is broadly classified into quantitative (types 1 and 3) or qualitative (type 2) abnormalities of plasma VWF [4]. Quantitative abnormalities include mild to moderately severe reduction of qualitatively normal VWF (type 1 VWD) or essential absence of VWF (type 3 VWD). Qualitative (type 2) VWD includes types 2A and 2B with deficiency of the high molecular weight (HMW) multimers and discordantly decreased VWF:RCo (compared to VWF:Ag), and type 2 M VWD with discordantly decreased VWF:RCo, but no substantial reduction of HMW VWF multimers [5]. Limitations of current technologies [6–9] preclude reliance solely on the VWF:RCo/VWF:Ag ratio in the diagnosis of type 2 VWD variants. Moreover, the relative excess of types 2A/B over 2 M VWD requires VWF multimer analysis for subtyping of type 2 VWD [1,10].

The multimeric nature of plasma VWF was reported in 1978 by Fass et al [11], and in 1980 Ruggeri and Zimmerman [12] and Hoyer and Shainoff [13] described autoradiographic visualization of plasma VWF multimers in agarose slab electrophoretic gels. Methods of VWF multimer analysis have evolved such that multimer visualization with radioiodinated antibody and subsequent autoradiography have been

Abbreviations: AVWS, acquired von Willebrand syndrome; DDAVP, desmopressin; FVIII:C, factor VIII coagulant activity; HMW, high molecular weight; LVAD, left ventricular assist device; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:Ag, VWF antigen; VWF:RCo, VWF ristocetin cofactor activity.

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largely replaced with colorimetric or luminescent immunostaining techniques. The latter entail agarose gel-blotting or electroblotting to transfer proteins to a membrane, followed by applications of primary antibody and secondary enzyme labeled antibody, with immunoenzymatic visualization of plasma VWF multimers [11,14–16]. However, non-uniform transfer of VWF multimers, from agarose gel to a membrane can cause erroneous VWF multimer interpretation due to inefficient blotting transfer of HMW VWF multimers [17–19]. Such problems would be resolved in principle by direct staining of gels. Herein, we describe a novel imaging technique that uses a secondary antibody labeled with a near-infrared fluorochrome to visualize VWF multimers directly in the agarose gels, obviating electroblotting or autoradiography. We validated this new method using normal pooled plasmas (commercial and internal sources) and individual plasma samples from selected patients with known VWD or acquired VWF abnormalities including some with AVWS.

Materials and Methods

Study population and sample collection

Archived plasma samples collected from established VWD patients followed in the Mayo Comprehensive Hemophilia Treatment Center were used for this study. VWD subtypes included type 1 (n = 8); type 2A (n = 8); type 2B (n = 7); type 2 M variant with ultra large molecular weight multimers (n = 6); type 2 variant (n = 1) and type 3 (n = 5). Criteria for diagnosis and typing of VWD conformed to the recommendations of the Scientific Standardization Committee on von Willebrand Factor of the International Society on Thrombosis and Haemostasis [5]. At the time of specimen collection, patients had not recently received treatment with desmopressin (DDAVP) or infusions of VWF concentrates or blood products. After obtaining informed consent, fasting blood samples were collected into evacuated tubes containing one-tenth volume 3.2% trisodium citrate. Platelet-poor plasma was prepared by centrifugation at 1,500 g for 10 minutes at 5 °C. Plasma samples were stored in small aliquots at -70 °C prior to thawing at 37 °C for testing.

A second set of 49 plasma samples with normal multimer patterns (designated as type I multimer pattern; n = 35) and abnormal multimer patterns with loss or reduction of HMW multimers (designated as type II multimer pattern; n = 14) was selected from samples referred to the Mayo Clinic Special Coagulation Laboratory for VWF multimer analysis (reference laboratory samples). No clinical information was available for these patient samples. These samples, along with samples from commercial pooled normal plasmas, were analyzed by both autoradiography and the in-gel infrared fluorescent immunostaining method for comparison. In addition, plasma samples from Mayo Clinic patients with chronic congestive cardiac failure and implanted left ventricular assist devices (LVADs) with acquired VWF abnormalities and type II VWF multimer patterns (n = 9) (Table 1) were analyzed by the in-gel infrared fluorescent immunostaining method. The Mayo Clinic Institutional Review Board approved this study.

Reagents and instruments

Reagents and instruments used in this study were purchased from USA sources, unless otherwise noted. Routine laboratory chemicals were from Sigma Diagnostics (St. Louis, MO), Fisher Chemical (Fairlawn, NJ), Baker (Phillipsburg, PA), or Bio-Rad (Hercules, CA). Other reagents included in-house or purchased normal pooled plasmas (FACT from George King Biomedical, Overland Park, KS or Cryocheck from Precision Biologic, Dartmouth, Nova Scotia, Canada); VWF:Ag automated latex immunoassay (LIA) kits (Instrumentation Laboratory, Lexington, MA or Diagnostica Stago, Parsippany, NJ); primary anti-human VWF antibody (polyclonal rabbit anti VWF) from Dako USA (Carpinteria, CA) [antibody A] or alternative sources (Gene Tex, Inc., Irvine, CA [antibody B] or Vector Laboratories, Burlingame, CA [antibody C]); secondary Alexa Fluor 680 goat anti-rabbit IgG (Invitrogen Molecular Probes, Eugene OR). VWF images were acquired and processed with an infrared imaging system (Odyssey, LI-COR Biosciences, Lincoln, NE).

Table 1
Characteristics of tested samples.

Sample types		Sample Number	VWF:Ag (IU/dL) Median (Range)	VWF:RCo* (IU/dL) Median (Range)
VWD Patients (n = 34)	Type 1	8	30(5–48)	38(<12–46)
	Type 2 A/B	15	54(43–182)	28(<12–55)
	Type 2 M	6	29(17–35)	12(<12–13)
	Type 3	5	<5	<12.5
Acquired VWF abnormalities		9	140(113-225)	120(96-205)
Reference lab samples		-	-	-
Type I ** multimer pattern		35	98(18-275)	86(<12-228)
Type II ** multimer pattern		14	40.5(<13-106)	27.5(<12-60)

* lower detection limit of VWF:RCo assay (<12.5 IU/dL). ** defined in methods section. Abbreviations: VWF, von Willebrand factor; Ag, antigen; VWF:RCo, ristocetin cofactor activity; VWD, von Willebrand disease.

Assays of factor VIII:C, VWF:Ag, and VWF:RCo

Factor VIII activity (FVIII:C) was measured by one stage clot-based assay with MDA-180(II) instrumentation (Trinity Biotech, Berkeley Heights, NJ) using mixtures of patient and FVIII-deficient substrate plasma (George King Biomedical), activated partial thromboplastin time (APTT) reagent (MDA Platelin L, Trinity Biotech), and CryoCheck plasma (Precision BioLogic) for calibration. VWF:Ag was determined by automated latex immunoassay (LIA) using ACL TOP IL (Instrumentation Laboratory) or STAR Evolution (Diagnostica Stago) instrumentation and reagents, and kit calibrators linked to `WHO standards. The assay for VWF:RCo, based on washed platelet aggregometry in the presence of ristocetin (ristocetin A sulfate, American Biochemical and Pharmaceutical, Marlton, NJ), was modified from the original method reported by Olson et al [20,21] including use of FACT calibrator plasma (George King Biomedical) and measurement with a Bio/Data (Horsham, PA) 4-channel aggregometer.

VWF multimer analysis

Preparation of plasma samples

Frozen plasma samples were thawed in a 37 °C water bath for 10 minutes and gently mixed by repeated sample tube inversion. Thawed plasma samples (10 μL) were diluted in 85 μL of sample prep buffer (0.01 mol/L Tris-HCl, 0.001 mol/L EDTA, pH 8.0, with 2% SDS) supplemented with 5 μL of 1% solution of sodium bromphenol blue, and incubated 15 minutes in a water bath at 60 °C. For plasma samples with VWF:Ag <20 IU/dL, 20 μL of plasma were diluted in 75 μL of 2X sample prep buffer (0.007 mol/L Tris-HCl, 0.0007 mol/L EDTA, pH 8.0, with 4.7% SDS) supplemented with 5 μL bromphenol blue, and incubated 15 minutes at 60 °C. Electrophoresis samples were then maintained at ambient temperature until transferring 20 μL aliquots into agarose gel sample wells.

Preparation of agarose gels

High gelling temperature (HGT) agarose (Seakem, FMC Bioproducts [Lonza Rockland, Rockland, ME]) was used to prepare a stacking