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Weibel-Palade body formation and exocytosis in von Willebrand disease
Wang, J.W.

Citation

Wang, J. W. (2013, January 17). *Weibel-Palade body formation and exocytosis in von Willebrand disease*. Retrieved from <https://hdl.handle.net/1887/20418>

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Author: Wang, Jiong-Wei

Title: Weibel-Palade body formation and exocytosis in von Willebrand disease

Issue Date: 2013-01-17

Chapter 2

Von Willebrand disease and Weibel-Palade bodies

Jiong-Wei Wang and Jeroen C. Eikenboom

Adapted from *Hamostaseologie*. 2010 Aug;30(3):150-155

Summary

Von Willebrand factor (VWF) is a pivotal hemostatic protein mediating platelet adhesion to injured endothelium and carrying coagulation factor VIII (FVIII) in the circulation to protect it from premature clearance. Apart from the roles in hemostasis, VWF drives the formation of the endothelial cell specific Weibel-Palade bodies (WPB), which serve as a regulated storage of VWF and other thrombotic and inflammatory factors. Defects in VWF could lead to the bleeding disorder von Willebrand disease (VWD). Extensive studies have shown that several mutations identified in VWD patients cause an intracellular retention of VWF. However, the effects of those mutations on the formation and function of its storage organelle are largely unknown. This review gives an overview on the role of VWF in WPB biogenesis and summarizes the limited data on the WPB formed by VWD-causing mutant VWF.

Introduction

Von Willebrand disease (VWD) is the most common bleeding disorder in humans with a prevalence of up to 1% in some populations [1]. It is caused by inherited defects of the plasma glycoprotein von Willebrand factor (VWF) [2]. Quantitative deficiency of VWF leads to type 1 or type 3 VWD, while functional defects lead to type 2 VWD. Type 1 VWD is the most common form, but type 3 VWD is the most severe [3].

VWF is a multimeric glycoprotein exclusively synthesized in endothelial cells and megakaryocytes, and stored, respectively, in Weibel-Palade bodies (WPB) and α -granules in the platelets [4,5]. In vivo, when the endothelium is activated or damaged, WPB serve as a rapid release pool of cytokines as well as VWF [6]. VWF plays important roles in the primary hemostasis by mediating the adhesion of platelets to the injured vascular wall, and in the secondary hemostasis by serving as the carrier of coagulation factor VIII (FVIII) [7].

Interestingly, VWF is also the key factor to drive the formation of WPB [8]. In the last two decades several mutations in the *VWF* gene have been identified in VWD patients, however, the impact of those mutations on the formation and function of WPB is still largely unknown. In this review, we discuss recent studies on quantitative VWF defects to understand the mechanisms underlying VWD and the limited data on WPB formation by VWD-causing VWF mutants.

Von Willebrand factor and Weibel-Palade bodies

The biosynthesis of VWF is a complex process and requires a series of posttranslational modifications (see for review [7,9]). In endothelial cells, VWF is synthesized in the endoplasmic reticulum (ER) as a 350 kDa monomer, the proVWF, composed of a signal peptide and 14 domains (D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK). Via disulfide bridges, the proVWF dimerizes at the C-terminus in the ER and the proVWF dimers multimerize at the N-terminus in the Golgi apparatus to form multimers of up to 20 MDa. During this process, VWF undergoes extensive modification, such as glycosylation and sulfation. In the Golgi apparatus, the proVWF is cleaved by furin into propeptide (D1-D2) and mature VWF. At the *trans* Golgi network (TGN), VWF is either immediately secreted constitutively or tubulized and stored into WPB for regulated secretion [7,9].

WPB were first described by Weibel and Palade in 1964 and used as a hallmark of endothelial cells [10]. Viewed by the electron microscope (EM), WPB are large (100~200nm in caliber and 1~5 μ m in length), high electron-dense cigar-shaped

organelles with regular internal striations [10]. When the endothelium is exposed to some stimuli, the WPB undergo exocytosis to release a body of cytokines such as P-selectin, angiopoietin 2, interleukin 8, tissue plasminogen activator (t-PA) and VWF to the circulation [6,11].

Role of VWF in the formation of WPB

VWF is not only stored in WPB but it also drives the formation of these vesicles [8]. Heterologous expression of VWF in several non-endothelial cell lines forms de novo storage vesicles of VWF, pseudo-WPB, resembling endothelial cell WPB [8,12-14]. On the other hand, in VWF deficient mice, pigs or dogs the endothelial cells lack WPB [15-17]. More over, these organelles can still be re-established by expressing VWF in the VWF-deficient canine aortic endothelial cells from VWD dogs [17].

Although the expression of VWF is necessary, it is not sufficient to drive the formation of WPB. Expression of VWF in some cell lines, including COS (monkey kidney cells) [8] does not result in the formation of pseudo-WPB. And the porcine aortic endothelial cells lack WPB although they do express VWF [16,18]. In megakaryocytes, VWF is stored in the α -granules which are virtually of round shape, instead of the elongated WPB [19]. Why these VWF-expressing cells are not able to form (pseudo-) WPB is unclear.

WPB are characterized as elongated organelles with longitudinally oriented VWF tubules closely wrapped by the membrane. Accumulating evidence supports the hypothesis that the tubulation but not the multimerization of VWF is required for the elongation of WPB [20]. Perturbation of intracellular pH with weak bases causes disruption of the tubular structures and loss of the elongated shape of WPB [21,22], conversely, perturbation of the multimerization by either disrupting the disulfide bonds formation or deleting the C terminus after A1 domain does not impair the tubulation of VWF or the shape of WPB [22]. This is confirmed by assembling the right-handed helix tubules in vitro, under low pH and calcium ions, with only pure propeptide and dimerized D'D3 domains [23].

Physiological role of VWF tubular storage in WPB

The tubular storage is supposed to compact VWF by 100-fold and determine the unique shape of WPB. The elongated shape of WPB may be essential to the physiological function of VWF because the hemostatic function of this protein relies on its storage format as highly compacted tubules [22]. The twisted tubular

striations observed by tomographic analysis indicate these tubules may be assembled by a “spring-loading mechanism” [24]. Upon exocytosis, it allows a rapid unfurling of VWF tubules into ultralong strings (up to 100 μm) docking on the endothelial cells to adhere the platelets [25]. Perturbation of the elongated shape prior to exocytosis yields short and tangled VWF strings with impaired ability to catch platelets both in vitro and in vivo [22]. Physiologically, these ultralong strings are cleaved rapidly by ADAMTS13 into smaller sized multimers [25,26], otherwise it may lead to depletion of platelets or thrombosis. Conversely, impaired secretion or assembly of VWF strings upon WPB exocytosis, as in VWD, may cause a bleeding tendency [3]. However, the secretion and assembly of VWF strings in VWD have not been studied yet.

All these processes of WPB formation are dependent on VWF structure and may be disrupted by mutations in VWF as detected in patients with VWD. Little is known about the effects of VWF mutations on the WPB formation.

VWF secretion and defects in VWD

At the TGN, VWF is directed either into constitutive or regulated pathways of secretion [27]. A more recent study argues that WPB are presumably responsible for both the regulated and basal secretion [28]. Impaired secretion of VWF caused by mutations in the *VWF* gene, leads to quantitative deficiency of VWF [3]. However, which secretion pathway is defected remains elusive.

VWF Mutations identified in VWD patients with quantitative VWF defects

The quantitative VWF deficiency is responsible for VWD type 1 (moderate deficiency) or type 3 (complete deficiency). Type 1 VWD is the most common form, but the underlying genetic mechanism remained unknown for a long time. Recently, three multicenter studies have now provided new insights into the molecular defects of type 1 VWD [29-31]. Candidate mutations were identified in 70% of 150 index cases in the European multicenter study (MCMMDM-1 VWD) [29], in 63% of 123 index cases in the Canadian study [30], and in 63% of 32 index cases in the UK study (UKHCDO) on type 1 VWD [31]. The mutations identified in these studies on type 1 VWD are extremely heterogeneous with a complex spectrum throughout the *VWF* gene, however, the large majority of patients are heterozygous for missense mutations (75%), and only 15% have null alleles. This is unlike type 3 VWD with about 80% of mutations predicted as null alleles [32]. Although some missense mutations have been identified in a minority of type 3 VWD patients,

most patients are homozygous or compound heterozygous for nonsense mutations, frameshifts, splice site defects, or large gene deletions [32] (Mutation database <http://www.vwf.group.shef.ac.uk/>).

Impaired constitutive secretion and intracellular retention in quantitative VWF defects

Plasma levels of VWF in VWD patients can be reduced by decreased synthesis, increased clearance, impaired secretion, some unknown factors, or the combination of them. In patients with null alleles the reduced levels of VWF can be easily understood because of the lack of VWF synthesis. The role of increased clearance of VWF in VWD is emerging. In human and mouse studies faster clearance of several VWF mutants has been demonstrated [33-36]. Measurement of the ratio between the propeptide and mature VWF may help identify the patients with increased clearance [34-36]. Also blood group O contributes to the type 1 VWD phenotype by reducing the VWF levels, possibly through more rapid clearance although the mechanism is still unresolved [29,37,38].

Impaired secretion and intracellular retention seem to be the major mechanism involved in patients with quantitative VWF defects associated with missense mutations. By expression of the full-length cDNA of recombinant VWF in COS-7 cells, we confirmed the pathogenic nature of 11 of 14 candidate missense mutations identified in type 1 VWD and the predominant pathogenic mechanism was intracellular retention [39]. Using various heterologous expression systems, we and others also confirmed reduced secretion as the pathogenic mechanism of several other mutations [40-49].

Those missense mutations may reduce the secretion of VWF by misfolding of the protein and ER-associated degradation [50]. Actually, the loss of high molecular weight multimeric VWF by a subgroup type 2A mutations was proposed due to the increased binding with ER chaperones [51]. The quantitative VWD-causing mutation p.Arg273Trp was shown in COS-7 cells to cause ER retention of VWF by prolonged association with two chaperones in ER, ERp57 and calnexin [52]. As many of the missense mutations in quantitative VWD involve either the loss or gain of a cysteine residue, we have evaluated the role of cysteines in the pathogenesis of quantitative VWD. Expression of six type 1 or type 3 VWD mutations with loss of cysteines involved in intrachain disulfide bonds, all showed an impaired secretion of VWF [41,42]. Our recent expression study of type 1 VWD mutations also showed that mutations with loss (p.Cys2257Ser, p.Cys2304Tyr and p.Cys2477Tyr) or gain

of cysteines (p.Gly2441Cys and p.Arg2464Cys) yielded similar defects [39]. However, loss of an interchain disulfide bond (p.Cys2773Ser) led to a dominant negative qualitative defect of VWF (type 2A, subtype IID) with normal secretion by transfected 293T cells [53]. The result is consistent with that of another change at the same position, p.Cys2773Arg [54]. The expression studies suggest that the impaired constitutive secretion by intracellular retention or degradation is a more general mechanism for the reduced VWF level in VWD patients with missense mutations, and cysteines involved in the intrachain disulfide bond formation may especially contribute to this mechanism.

WPB formation, structure and regulated secretion in VWF defects

Up to date, the pathogenic mechanisms of many VWD-causing VWF mutations have been only partially revealed. As the most prothrombotic form of VWF is stored for regulated secretion in WPB [55,56], defects in WPB formation and function, if any, caused by the VWF mutations in VWD, may be very important. Due to the limited access to VWD endothelial cells and the limitation of suitable heterologous cell systems the studies on the WPB in VWD are very limited (see Table 1).

Studies in heterologous systems

Non-endothelial cells have been used for the evaluation of the effects of VWD-causing mutations on the WPB formation and regulated secretion (Table 1). Expression of a type 2A causing mutation p.Cys1234Trp in AtT-20 cells, led to a mild defect in multimerization and constitutive secretion, but a nearly normal intracellular storage of VWF [49]. Although the regulated secretion of the pseudo-WPB was not investigated in that study, the normal intracellular storage is consistent with the good response to DDAVP infusion in the patients [49]. In the same study, the mutation p.Cys1157Phe (unclassified VWD) showed abolished multimerization, severely impaired constitutive secretion but normal storage of VWF [49]. A more recent study showed that an in-frame deletion D437-R442del within the VWF propeptide D2 domain, with loss of VWF multimerization, completely abolished the pseudo-WPB formation in AtT-20 cells [57].

Expression of VWF in AtT-20 cells may be complicated because of endogenous secretory organelles and the fact that VWF storage vesicles are more round [14]. For example the p.Tyr87Ser mutation with overabundant dimeric VWF in plasma shows abolished multimerization but storage in VWF positive round granules in AtT-20 cells, suggesting normal storage [48]. However, expression of p.Tyr87Ser in

HEK293 cells, in which pseudo-WPB are usually elongated, showed only round storage vesicles and overexpression in human umbilical vein endothelial cells (HUVECs) shortened pre-existent WPB [22]. This indicates that p.Tyr87Ser disrupts tubulation of VWF and thus elongation of WPB.

Emerging evidence indicates HEK293 cells may be a better heterologous system to analyze the defects caused by VWF mutations, in particular the regulated storage of VWF. Expression of VWF in HEK293 cells induces formation of VWF storage vesicles, pseudo-WPB, indistinguishable from real WPB, based on storage of multimeric VWF; elongated shape with a size of 100~200 nm by 1~5 μm ; containing internal striations which are VWF tubules; recruitment of appropriate membrane proteins on WPB; and secretion of stored VWF upon stimulation [14,58]. In this system, Michaux et al. [14] studied the quantitative mutant p.Arg273Trp, which showed shorter pseudo-WPB but normal recruitment of the membrane proteins CD63 and P-selectin and dilated ER, suggesting delayed exit of VWF from ER. Two type 2N VWD mutants, p.Cys1225Gly and p.Cys788Arg, led to a relatively mild defect in the pseudo-WPB formation. Empty spaces possibly due to impaired condensation or disrupted tubule alignment were observed by EM between the tubules and the membrane in some elongated pseudo-WPB [14]. Stimulating the cells expressing each of the 3 mutants with phorbol ester showed an impaired regulated secretion of VWF compared to cells expressing wild-type VWF [14].

Studies in endothelial cells

The most optimal model to study the VWF defects would be endothelial cells. The earliest study on the intracellular storage of VWF in VWD is from Booyse and coworkers [59] who cultured HUVECs from a child with VWD. Compared with normal HUVECs the VWD HUVECs demonstrated a distinct pattern of VWF distribution with much fewer “intensely stained granules” and “fine diffuse material” in the perinuclear region of the cells [59]. The latter probably reflects VWF retained in the ER. Another study with cultured HUVECs from two type 1 VWD patients showed diminished constitutive secretion and regulated secretion accompanied with a two- to four-fold reduction in VWF mRNA [60]. EM analysis of one type 1 VWD umbilical vein segment illustrated that the morphology of WPB appeared normal, however, the diminished releasable pool of VWF in the cultured VWD HUVECs indicated a likely decreased storage of VWF [60]. Unfortunately, the genetic defects of these patients are not available. The HUVECs from a type 2A

Table 1. (Pseudo-) Weibel-Palade bodies formed by VWD-causing VWF mutants

VWD type	Mutation	Cell system	Storage organelle	Cigar shaped	Number of organelles	Regulated secretion	Membrane protein	Refs.
unclassified	p.Tyr87Ser	AtT-20	Yes	No	N.R.	N.R.	N.R.	[48]
		HEK293	Yes	No	N.R.	N.R.	N.R.	[22]
		HUVEC	Yes	No ¹	N.R.	N.R.	N.R.	[22]
		Carine VWD-AEC ²	Yes	N.R.	N.R.	Yes	Yes	[17]
1 or 3	p.Arg273Trp	HEK293	Yes	Yes	Reduced	Yes ³	Yes	[14]
2A	D437-R442del	AtT-20	No	N.A.	N.A.	N.A.	N.A.	[57]
		AtT-20 and RIN 5F	No	N.A.	N.A.	N.A.	N.A.	[64]
2N	p.Arg763Gly	CV-1	Yes	Yes	Normal	N.R.	N.R.	[12]
		HEK293	Yes	Yes	N.R.	N.R.	Yes	[65]
2N	p.Cys788Arg	HEK293	Yes	Yes	Reduced	Yes ³	Yes	[14]
2N	p.Thr791Met	HEK293	Yes	Yes	N.R.	N.R.	Yes	[65]
2N	p.Arg816Trp	HEK293	Yes	Yes	N.R.	N.R.	Yes	[65]
		AtT-20	Yes	N.R.	Normal	N.R.	N.R.	[66]
2N	p.Arg854Gln	HEK293	Yes	Yes	N.R.	N.R.	Yes	[65]
2N	p.Cys1060Arg	HEK293	Yes	Yes	N.R.	N.R.	Yes	[65]

Table 1. (Pseudo-) Weibel-Palade bodies formed by VWD-causing VWF mutants (continued)

VWD type	Mutation	Cell system	Storage organelle	Cigar shaped	Number of organelles	Regulated secretion	Membrane protein	Refs.
1 or 2N	p.Arg924Gln	AtT-20	Yes	N.R.	Normal	Yes	N.R.	[63]
		BOEC ⁴	Yes	Yes ⁵	N.R.	N.R.	N.R.	[63]
Unclassified	p.Cys1157Phe	AtT-20	Yes	N.R.	Normal	N.R.	N.R.	[49]
2N	p.Cys1225Gly	HEK293	Yes	Yes	Reduced	Yes ⁶	Yes	[14]
2A	p.Cys1234Trp	AtT-20	Yes	N.R.	Normal	N.R.	N.R.	[49]
Unknown	Unknown	HUVEC	Yes	Yes	Reduced	N.R.	N.R.	[59]
1	Unknown	HUVEC	Yes	Yes	Reduced	Yes ³	N.R.	[60]
1 ⁷	Unknown	HUVEC	Yes	Yes	Reduced	Yes ³	N.R.	[67]
2A	Unknown	HUVEC	Yes	Yes	Yes	N.R.	N.R.	[61]
2B	Unknown	HUVEC	Yes	Yes	Yes	Yes	N.R.	[68]
2N	HPP-VWF ⁸	AtT-20	Yes	N.R.	Normal	N.R.	N.R.	[66]

N.R.: Not reported; N.A.: Not applicable.

¹High expression of p.Tyr87Ser in HUVECs shortened the pre-existent WPB; ²Canine VWF-deficient aortic endothelial cells; ³Severely decreased; ⁴The BOECs derived from a compound heterozygous patient with a type 2N mutation p.Arg816Trp; ⁵Abnormally large (up to 3-fold); ⁶Mildly decreased; ⁷Type 1 VWD subtype "platelet low";

⁸HPP-VWF (Hereditary persistence of preproVWF): bearing two mutations (p.Arg782Trp and p.His817Gln) and a dinucleotide mutation [66,69].

VWD patient formed likely normal WPB [61].

HUVECs from VWD patients are not readily available. A promising alternative could be blood outgrowth endothelial cells (BOECs, also called late outgrowth endothelial cells) from patients' peripheral blood. These cells have endogenous WPB which release VWF under stimulation indistinguishable from HUVECs [62]. Very recently a study was published that has analyzed BOECs from a compound heterozygous patient with p.Arg816Trp (a type 2N VWD mutation) and p.Arg924Gln (a VWF variant frequently identified in type 1 VWD patients). Abnormally large WPB were seen compared to BOECs from a healthy control [63]. The mechanism underlying the formation of abnormally large WPB is unknown, and unfortunately the regulated secretion of these organelles was not studied. We have obtained BOECs from several VWD patients and the studies indicate that VWD-derived BOECs can be a useful tool to investigate the pathogenic role of VWF mutations (**Chapter 6 and 8**).

Conclusions and future perspectives

Many VWF mutations have been identified in VWD patients. Although several mutations were shown to cause impaired secretion and intracellular retention, the exact pathogenic nature of most of these mutations remains unclear. Investigation of the effects of these mutations on the constitutive secretion, WPB formation, structure and regulated secretion in a suitable heterologous system such as HEK293 cells or patient-derived BOECs may yield more insights.

Acknowledgement

J.W. Wang is financially supported by a grant from the China Scholarship Council (2007U21083).

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