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Endothelial Cell von Willebrand Factor Secretion in Health and Cardiovascular Disease

Luiza Rusu and Richard D. Minshall

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Abstract

The main function of von Willebrand factor (vWF) is to initiate platelet adhesion upon vascular injury. The hallmark of acute and chronic inflammation is the widespread activation of endothelial cells which provokes excessive vWF secretion from the endothelial cell storage pool. The level of vWF in blood not only reflects the state of endothelial activation early on in the pathogenesis, but also predicts disease outcome. Elevation in the blood level of vWF occurs either by pathologic increase in the rate of basal vWF secretion or by increased evoked vWF release from dysfunctional/activated endothelial cells (ECs). The increase in plasma vWF is predictive of prothrombotic complications and multi-organ system failure associated with reduced survival in the context of severe inflammatory response syndrome, type II diabetes mellitus, stroke and other inflammatory cardiovascular disease states. This chapter focuses on the role of high circulating vWF levels in thrombotic and inflammatory disease while paying attention to the emerging vWF-related drug development strategies.

Keywords: cardiovascular disease, endothelial activation, von Willebrand factor, hemostasis, thrombosis, inflammation, ADAMTS-13, vWF-related therapeutical strategies

1. Introduction

Endothelial dysfunction, often as a result of chronic activation, is a primary determinant of the severity of disease states. Apart from other cell-specific defects, activation of endothelial cells (ECs) increases platelet binding to von Willebrand factor (vWF), a multimeric blood protein primarily synthesized, stored and secreted by ECs [1–6]. Under physiological conditions, vWF is secreted from ECs via two pathways that enable hemostasis; a continuous or basal secretory pathway that maintains a baseline blood vWF level, [7] and the other, a regulated secretory

pathway induced by agonists such as thrombin [8, 9]. Basal vWF secretion involves elements of regulated secretion, with vWF targeted to Weibel-Palade bodies (WPBs) after Golgi processing, but continuously secreted from the storage pool [7]. Release of WPB contents into the extracellular space upon thrombin stimulation of ECs, thought to be pathophysiologically relevant in inflammatory cardiovascular disease, occurs via several processes: (1) signaling via $[Ca^{2+}]/G\alpha_q$ and $G\alpha_{12}/\alpha$ -soluble NSF attachment protein (α -SNAP) [10] that (2) promotes engagement and activation of vesicle (v) and target (t) membrane-associated v-SNARE and t-SNARE proteins (vesicle- and target-soluble NSF attachment protein receptors) [11, 12] and (3) energy-dependent fusion of WPB with plasma membrane that promotes the formation of a secretion pore through which the exocytosis of WPB contents occurs [13–15].

Once secreted from WPBs, vWF circulates in a globular conformation under resting conditions [16] and is a carrier for circulating coagulation factor VIII, thereby protecting it from degradation [17–19]. The blood vWF level in health and inflammatory disease is predominantly EC derived, while the contribution of platelets is rather minimal [20]. In response to high shear stress and inflammatory mediators [21], normally quiescent ECs secrete long vWF multimers in large quantities from the storage pool into systemic circulation. vWF multimers are then extracellularly cleaved by a metalloprotease ADAMTS-13 (A Disintegrin and Metalloproteinase with a thrombospondin type 1 motif, member 13), unfold in the circulation and self-associate into particularly linked concatemers to form ultra-large vWF strings [22]. Platelets spontaneously bind to activated vWF via glycoprotein Iba ($GpIb\alpha$) interaction with the exposed A1 domain, initiating the thrombogenic process [23–26]. In addition to its classical role in platelet binding, the vWF A1 domain also plays a role in vWF-dependent inflammatory responses [27–29]. In this chapter, we will discuss the most recent findings related to vWF secretion from ECs, the finely tuned balance of vWF level and activity in hemostasis and thrombosis, and the mechanisms by which vWF/ADAMTS-13 axis links inflammation and thrombosis. The potential of vWF as a target in antithrombotic therapies is also discussed.

2. Molecular mechanisms of vWF secretion

2.1. von Willebrand factor and Weibel-Palade bodies at a glance

WPBs are unique secretory granules in ECs, which were first discovered in the 1960s by transmission electron microscopy [30]. Later on, vWF was identified inside WPBs of cultured human ECs [4, 5, 31]. vWF synthesis and maturation trigger WPB biogenesis [32, 33]. vWF is the best example of a protein that drives its own organelle formation [5, 34, 35]. To date, the structure of vWF consists in domains arranged as follows: D1D2D'D3A1A2A3D4C1C2C3C4C5C6CK. Of these, D1D2 is the pro-peptide and, the rest, from D'-CK is the fully functional, mature vWF [36]. The high density of vWF in the secretory granules enables a bolus of fully functional vWF to be released at the site of vascular injury or inflammation, which will promote efficient platelet binding and hemostasis or lead to thrombosis [37]. The physiological importance of proper WPBs formation can be appreciated in the context of various disease states associated with the release of immature, defective or low levels of VWF due to altered Golgi multimerization or defective tubular packing in WPBs (i.e. type 2B von Willebrand disease (vWD)) [38, 39].

WPBs originate from clathrin and clathrin adaptor-protein1-coated vesicles, [35, 40] which, when loaded with vWF cargo, bud from the trans-Golgi network, continue to mature and vWF contained in them becomes densely packed and highly multimeric vWF [35]. Once the vesicles had formed, they lose the clathrin coat, and the interactions between WPBs and the actin cytoskeleton are thought to either tether WPBs to the perinuclear cytosolic compartment or to relocate WPBs in the proximity of the plasma membrane in preparation for fusion and exocytosis [41]. Upon secretion of its contents, WPBs dissociate from the membrane and are recycled and refilled [35].

Interestingly, vWF secretion is influenced by plasma sodium level [42]. In mice subjected to mild water restriction, elevated endothelial vWF protein levels correlated with increased number of micro thrombi in capillaries [42]. Hypernatremia causes up-regulation of vWF gene expression via nuclear factor of activated T-cells 5 (NFAT5) binding to the vWF promoter [42]. Another regulator of vWF was found to be autophagy [43]. Endothelial cell-specific ATG7 (critical factor in autophagy) deficient mice have prolonged bleeding times as compared to control, and decreased vWF secretion of immature vWF [43].

At least two different secretion mechanisms of WPB cargo have been reported so far: selective, time-dependent 'kiss-and-run' release [13] and differential release of the distinct components of these storage granules [8]. Apart from vWF, WPBs also contain other inflammatory and vasoactive mediators: P-selectin, Rab-27, coagulation factor XIIIa, tissue plasminogen activator, interleukin-8 (IL-8), eotaxin, endothelin-1, endothelin-converting enzyme, calcitonin-gene-related peptide, α 1,3-fucosyltransferase VI and angiopoietin 2 [44]. vWF and the other components—stockpiled in and liberated as needed from WPBs—provide the ECs with the ability to react acutely to restore homeostasis; vWF plays a role in hemostasis and thrombosis, and also, in the regulation of inflammation [45], vascular permeability [27] and angiogenesis [46].

vWF is synthesized exclusively by ECs, megakaryocytes and platelets and stored in specialized secretory granules. EC WPBs and platelet α -granules share several characteristics, as both organelles contain vWF, P-selectin and CD63 [47, 48]. But, in platelets, vWF detection by immune-gold labeling is restricted to a subset of spherical α -granules [48]. In this vane, spherical secretory α -granules are quite distinct from WPBs. First, the vWF content of platelets is relatively low: 0.05–0.1 U per 3×10^8 platelets [49]. For comparison, plasma vWF concentration is 1–1.8 U/mL (10 μ g/mL blood), of which only 15% of plasma vWF comes from platelets, while the majority 85% of the total vWF comes from ECs [4, 20, 34, 50]. Second, ECs combine basal and regulated vWF release, while platelets do not have a mechanism of basal secretion and, thus, substantial platelet activation would be needed for platelet vWF secretion to significantly contribute to the elevated blood vWF observed in different disease states. Finally, excessive secretion of EC-derived vWF in inflammation is attributed to the large capacity of ECs to make and store vWF in EC-specific storage pool, that is, in WPBs.

Thus, EC secretory granules supply the vasculature with circulating vWF to control hemostasis and thrombosis, while platelets play only a secondary role in this process [20, 49]. A balanced level of EC-derived vWF is critical to overcome the thrombotic complications in pathological settings, therefore it is important to understand the underlying endothelial constitutive and evoked exocytosis mechanisms which provide the bloodstream vWF in health and disease.

2.2. Basal versus regulated vWF secretion

Secretion of vWF occurs by one of the following three modes:

- 1 regulated secretion from WPBs, in response to a specific agonist (secretagogue) on-demand upon vascular injury [51, 52];
- 2 basal (constitutive-like or continuous) secretagogue-independent secretion of vWF from WPBs [7];
- 3 constitutive, secretagogue-independent release of vWF from non-WPB compartments [34, 40].

Over the years, there have been in the field opposite views about how much vWF is released through each of these three pathways and what determines the balance between basal (constitutive-like) and regulated vWF secretion is still under debate [34, 53, 54].

2.2.1. Basal vWF secretion

In resting ECs, secretion of vWF was thought to take place predominantly via conventional constitutive vWF secretion which is susceptible to protein synthesis inhibitor cycloheximide [34]. However, the importance of this pathway was exaggerated initially, as it could be drawn from the more recently published studies listed below. A vWF multimeric analysis in ECs challenged this view, indicating that highly multimeric vWF might be released solely from WPB storage pool [53]. Most recently, the issue was revisited when a study using metabolic labeling of vWF in human umbilical vein ECs (HUVECs) showed that most of the vWF secreted from unstimulated cells is not mediated by conventional constitutive secretion, but rather by basal release from the WPBs [7, 55]. Giblin and colleagues [7] recently brought more evidence to dismiss the belief that multimeric vWF passes uninterrupted from the Golgi to plasma membrane. They showed that freshly synthesized vWF is held for at least 24 h in WPBs before unstimulated release occurred, escaping cycloheximide-induced inhibition of protein synthesis, and, thus, consistent with release from the storage pool [7]. Similarly, Romani de Wit and coworkers [56] studied the dynamics of GFP-vWF-positive WPBs in resting HUVECs over time, and they were able to capture multiple image frames of WPBs traveling long distances toward the cell periphery. Intriguingly, resting ECs displayed membrane-apposed accumulation of GFP-vWF-containing vesicles [33, 56, 57]. It is worth mentioning here that only whenever a component of the cytoplasmic machinery is ablated the constitutive vWF secretion becomes predominant [40]. Taken together, these studies of WPBs behavior suggest that only the multimeric form of vWF is stored and that ECs secrete multimeric vWF via a continuous secretory pathway, which is a source of vWF in the bloodstream. If the WPB storage compartment is the source of the continuously secreted form of vWF that travels in the bloodstream, then the ability to regulate this constitutive vWF secretory route has significant clinical implications. Disruption of continuous vWF secretion may be implicated in the pathophysiology of type 2 vWD, which is due to both reduced vWF-dependent platelet function and reduced highly multimeric vWF in plasma.

2.2.2. Regulated vWF secretion

The release of vWF from the regulated pathway occurs only following stimulation of ECs with an appropriate agonist, providing the endothelium with the means to react to its microenvironment by finely tuning the rate of release.

The notion that blood collected under stress clots faster was known for a while. In the 1970s, it was found that desmopressin (a synthetic analog of vasopressin) can elevate vWF level twofolds in healthy individuals [58]. The follow-up studies showed that desmopressin activates vasopressin receptor 2 (V2R) on ECs and triggers cyclic adenosine monophosphate (cAMP)-mediated release of vWF from EC WPBs [58]. A multitude of agonists have been reported to induce vWF release via regulated secretion. These include thrombin [51], calcium ionophores [34], hypoxia [59], vasopressin [58], histamine [14], complement [60], platelet-activating factor [61] and interleukins [62]. Importantly, high shear stress is modulating regulated vWF release [63].

2.3. G protein signaling in hemostasis and thrombosis

In order to understand how to control WPB secretion for therapeutic purposes, the signaling mechanisms of vWF secretion need to be elucidated.

The exocyst is a cytoplasmic protein complex which targets secretory granules from the trans-Golgi network to the plasma membrane; it facilitates docking and priming of the secretory granule to the plasma membrane, prior to SNARE-mediated fusion [64]. When WPBs are trafficked to the plasma membrane, the vesicles are aligned such that v-SNAREs from the vesicles and t-SNAREs from target membrane can assemble as α -helix zippers which pull the membranes together. This SNARE zipper model was studied in detail in synapses [65]. Interestingly, all major players of these complexes identified in neurons are also present in the endothelium, including SNAP23, syntaxin 2, 3 and 4, and vesicle-associated membrane protein 3 (VAMP3) complex which are thought to regulate vWF exocytosis [11].

Release of WPBs contents into the extracellular space is thought to occur via GTPase-dependent processes [10]. It has been postulated that G proteins mediate cell type and signaling microdomain-specific functions. According to the classic G protein signaling model, heterotrimeric G proteins are located in the proximity of the plasma membrane where they can be activated by seven transmembrane spanning receptors, the canonical G protein-coupled receptors (GPCRs), to provoke downstream signaling events. G proteins are GTPases that typically function through GTP hydrolysis and cycling between nucleotide free GDP-bound and GTP-bound forms. GTPases also control the timing and specificity of vesicle trafficking and the exocyst partners recognition events, without GTP hydrolysis [66, 67]; the distinction between cycling and non-cycling GTPases might be more obvious when examining the effect of GTP hydrolysis-deficient mutant proteins that would be expected to cause gain-of-function on non-cycling GTPases and loss-of-function on cycling GTPases [66]. Furthermore, several studies indicate heterotrimeric G proteins can rapidly shuttle between the plasma membrane and intracellular membranes to exercise their function upon cell-specific organelles, along the

secretory routes. Activation of GPCRs and G protein α and $\beta\gamma$ subunits of Gs, Gi, Gq/11 and G12/13 can stimulate secretory granule release [10, 68]. It has been shown, for example, by fluorescence polarization, that G protein i/o $\beta\gamma$ subunit competes with synaptotagmin for specific interaction sites on t-SNAREs, namely syntaxin 1 and SNAP25B (Synaptosomal-associated protein 25) [69]. G protein i/o binds to the SNARE at the plasma membrane, but in the presence of synaptotagmin and calcium, inhibits vesicle fusion with the plasma membrane, suggesting the G $\beta\gamma$ -SNARE axis has an inhibitory role during synaptic exocytosis [69]. In the exocrine pancreas, G proteins have been shown to play a role in early transport events [68].

A role for G proteins G α q/11 and G α 12/13 in hemostasis has been previously reported [70, 71]. Platelets from G α q-deficient mice fail to respond to low doses of platelet-activating agonists and these mice have prolonged bleeding times and reduced thrombus formation after intravenous administration of adrenaline/collagen [71, 72]. Studies in megakaryocyte-restricted G α 12/13 double knockout mice reveal that these mice have prolonged bleeding time and reduced thrombus formation, suggesting that G α 13-dependent signaling in platelets is also relevant for hemostasis and thrombosis [70]. Interestingly, we found that, in addition to G α s stimulatory-G protein [58, 73], G α 12 and G α q/11 facilitate exocytosis of vWF from ECs [10]. Importantly, in G α 12 overexpression studies, G α 12 is able to localize to the plasma membrane because of its palmitoylation at cysteine 11 [74]. Palmitoylation targets G α 12 to lipid rafts fractions [75]. Both wild-type G α 12wt and G α 12QL (constitutive active) were observed in lipid rafts fractions, and therefore, the localization of G α 12 to discreet endothelial microdomains may be independent of G α 12 activation [76]. Other studies reported direct G protein-dependent regulation of the SNARE protein fusion machinery is required for secretory granule exocytosis [77], and G α 12 was shown by the yeast two-hybrid method to interact with a member of the exocytotic assembly, α -SNAP [66]. Using cultured human ECs and knockout mouse models, we showed that depletion of G α 12 or α -SNAP inhibited both basal and thrombin-induced vWF secretion and that G α 12^{-/-} mice exhibit mildly reduced blood levels of vWF, but intact vWF multimeric pattern, and impaired thrombus formation [10]. Our studies suggest that G α 12 may interact directly with α -SNAP to promote the docking and fusion of WPBs [10]. Furthermore, G α 12 and G α q subunits, which are known to regulate actin cytoskeleton rearrangements in ECs via activation of RhoA GTPase, promote WPB docking on the plasma membrane, providing both direct and indirect mechanisms linking GPCR activation and SNARE complex fusion [10].

2.4. Kinetics of WPB secretion

Secretion of vWF from ECs is mediated by fusion of WPBs with the plasma membrane in a manner dependent on the ATPase N-ethylmaleimide-sensitive-factor (NSF), soluble-NSF-attachment protein alpha (α -SNAP) and SNAREs [78]. NSF binds to SNARE complexes to facilitate the disassembly of the zippered bundles [79]. Because NSF lacks a direct binding domain for members of the SNARE family, it connects via an adaptor, α -SNAP [80]. Six NSF proteins assemble together at the plasma membrane, and each NSF hexamer requires three α -SNAPs to mediate binding to the SNAREs [78]. Once the NSF/ α -SNAP/SNARE complex is formed, NSF hydrolyses ATP, providing the energy necessary for the disassembly of the

SNARE complex [81]. Therefore, the rate of exocytosis depends on α -SNAP and NSF activity [78]. Using PC12 cells as an α -SNAP-regulated exocytosis model, it was shown that α -SNAP, in absence of NSF activity, can actually block exocytosis and that this α -SNAP-dependent event occurs by direct binding of α -SNAP to free syntaxin, thus preventing SNARE complex assembly [82]. It is documented that α -SNAP is activated in the exocyst complex by phosphorylation [83], that α -SNAP binds and stimulates NSF ATPase activity, [84] that $G\alpha_{12}$ interacts with α -SNAP, [66] and that S-nitrosylation of NSF inhibits WPB exocytosis [85]. α -SNAP regulates exocytosis of granules from different types of cells [86]. α -SNAP binding to the SNARE complex in the fused membrane mediates recruitment and activation of NSF resulting of exocytosis from ECs [10, 87]. The $G\alpha_{12}$ binding site for α -SNAP was recently identified by using a library of substitution mutants within myc-tagged $G\alpha_{12}QL$ in which regions of the cDNA encoding consecutive six aminoacids were replaced with a sequence encoding the following six aminoacids: asparagine-alanine-alanine-isoleucine-arginine-serine (NAAIRS) by oligonucleotide-directed mutagenesis and expressed in a cell line as described previously [88], followed by evaluation of direct binding between $G\alpha_{12}$ NAAIRS mutants and α -SNAP by glutathione S-transferase (GST) pulldown [10]. Based on the evidence generated by the GST pulldown assay, we constructed an α -SNAP Binding Domain peptide to which we added a myristoyl group and micellar nanoformulation for cellular entry, to further assess the role of $G\alpha_{12}$ interaction with α -SNAP in vWF secretion [89, 90].

2.5. Spatial and temporal regulators of the exocyst complex

Secretion of vWF from ECs is mediated by small GTPases via both second messengers Ca^{2+} and cAMP-dependent signaling pathways [91–93]. Small GTPases are the effectors of the exocyst complex, involved in temporal coordination, spatial segregation and proof-reading of membrane trafficking events [94]. Rab and Ral GTPases are thought to be involved in vesicle tethering, whereas RhoGTPases are spatial regulators of the exocyst complex [95].

RalA was the first GTPase found to co-sediment with WPBs in density gradients [96]. Ral A is activated by its exchange factor, Ral guanine-nucleotide dissociation stimulator (RalGDS) [97], which is kept inactive by β -arrestin under static conditions [98]. Upon GPCR activation, Ral GDS uncouples from β -arrestin and functions in its GTP-bound state. RalGDS downregulation with siRNA in thrombin-stimulated HUVECs leads to accumulation of WPBs in the proximity of the plasma membrane, but exocytosis is incomplete, suggesting RalGDS/ β -arrestin complex is necessary to link thrombin receptors to WPBs exocytosis [98]. Ral A facilitates WPBs trafficking and delivery to the plasma membrane [91]. Ral A has been implicated in actin cytoskeleton dynamic rearrangements through its direct interactions with effectors filamin A and RalBP1/RLIP76 (a RAC/CDC42 guanine-nucleotide activating protein (GAP) [99]. RalBP1 links RhoGTPases and RalGTPases, and, of note, RalBP1 GAP has an ATP binding domain, although it is not clear whether this domain is a motor required for assembly of the exocyst [99]. Studies conducted in our laboratory suggest that human pulmonary artery ECs treated with filamin A siRNA attenuates constitutive and thrombin-induced vWF secretion [100]. It has been proposed that Ral A/ Ca^{2+} -dependent signaling might be a prerequisite for the exocytotic machinery [101].

The second point of intervention for the Rab and Ral GTPases is anchoring and fusion of WPBs with the plasma membrane [8]. Ral A also promotes exocytosis by increasing phospholipase D1 (PLD1) activity and subsequent phosphatidic acid (PA) production, thereby facilitating plasma membrane fusion as described in more detail in Section 2.6 of this chapter. The Rabs are a family of over 60 members of small GTPases that control membrane identity and the actions of the intracellular vesicles; each type of secretory vesicle in the cells has a unique set of Rab family members, as reviewed in [102]; of these, six Rab proteins were found in association with WPBs: Rab3 isoform b [103], Rab3 isoform d [104], Rab27a [105], Rab3 isoform a, Rab15, Rab33a, Rab37 [106] and Rab35 [107]. Rab 27a is located on the cytosolic face of WPBs, is used as a marker of organelle identity, and has multifunctional capacities: it could either mildly inhibit the secretion of WPBs by associating with its effector MyRIP (myosin VIIa and Rab-interacting protein), thus anchoring WPBs to actin filaments and keeping them from attaching to the plasma membrane until the right timing, [57] or it could strongly activate the secretion of WPBs by associating with an alternate effector, synaptotagmin-like protein 4 (or granuphilin) (Slp4-a), [108] which links the secretory granules to the plasma membrane via syntaxin-binding protein 1 and syntaxin 2 and 3 [108, 109]. The ratio of Rab27a occupancy by Slp4-a or MyRIP dictates whether Rab27a is stimulatory or inhibitory with regards to WPB exocytosis [103]. Furthermore, Rab27 was shown to work synergistically with Rab15 to control exocytosis; Zografou et al. recently showed that simultaneous knockdown of the two Rabs using siRNA in HUVEC leads to a greater reduction in vWF secretion compared with knockdown of either Rab alone [106]. Their experiments further showed that Munc13-4, a known effector of Rab27a, co-localized with Rab15 on WPBs. The three proteins, Rab27a, Rab15 and Munc13-4, thus form a complex and work in tandem to help regulate exocytosis of vWF [106]. Rab 33b has an inhibitory effect on vWF secretion [106].

A recent genome-wide screen identified a completely new signaling pathway associated with WPB exocytosis. Rab 35, which is controlled by Rab GAP TBC1D10A, promotes ACAP2 (ArfGAP with coiled-coil, Ank repeat and pleckstrin homology domain-containing protein) activation, which inhibits histamine-induced Ca^{2+} -dependent vWF and P-selectin expression in human ECs. This study used constitutively active mutants of Rab35, downregulation with siRNA and a fluorescence activated cell sorting (FACS)-based vWF secretory assay to prove that Rab35 promotes histamine-induced vWF secretion in a TBC1D10A- and ACAP2-dependent manner [107]. Of note, ACAP2 GAP targets Arf 6 which is a positive regulator of vWF secretion from human ECs, as shown by total internal reflection fluorescence (TIRF) microscopy and FACS-based vWF secretory assay [107]. Arf 6 GTPase activity at the plasma membrane elevates phosphatidylinositol 4,5-bisphosphate (PI (4,5) P₂) levels via PI (4)P5-kinase activation, acting antagonistically to Rab35 through TBC1D10A Rab GAP [107]. Finally, among the Rabs found to be in association with WPBs, only Rab27 is known to be involved in basal secretion [57, 108].

2.6. Endothelial cell secretory microdomains

GTPase-mediated exocyst activity of the SNARE assembly occurs in specific regions of the plasma membrane with distinct lipid profiles [110]. Namely, we know there is an increase in

PA production in HUVECs upon stimulation with histamine, which is a known agonist to induce exocytosis of WPB contents [111]. The increase in PA production is mediated by recruitment and activation of PLD1, an enzyme that hydrolyses phosphatidylcholine to produce PA [111]. PLD1 downregulation using shRNA resulted in a reduced secretion of vWF upon histamine stimulation [111]. PLD1 is commonly thought of as a general promoter of membrane fusion because of its role in producing fusogenic conical lipids such as PA [112]. PLD1 requires activation by one or more factors specific to the cell type and activation pathway, including small GTPases such as those of the ADP-ribosylation factor/Rho families as well as RalA, RalGDS or protein kinase C [113]. Thus, RalA and RalGDS not only play a role in the exocytosis process itself, as discussed in Section 2.5, but are also directly associated with the cytosolic face of WPBs. Therefore, it has been suggested that RalA could serve as an upstream activator of PLD1, promoting PLD1 movement to the membrane and subsequent generation of PA-enriched membrane microdomains important for membrane fusion [97].

2.7. Zyxin and other proteins that regulate vWF release from endothelial cells

Data published by Han et al. show that zyxin, a focal adhesion LIM domain-containing protein, is involved in thrombin-mediated remodeling of the actin cytoskeleton [114]. The molecular structure of zyxin predicts its function in cytoskeletal dynamics [115]. Zyxin has proline-rich repeats at the N terminus followed by a leucine-rich nuclear export signal (NES) and three copies of a cysteine- and histidine-rich motif called the LIM domain at the C terminus [115]. Regulators of cytoskeletal dynamics, such as Enap/vasodilator-stimulated phosphoprotein (VASP) family members and α -actinin interact with the proline-rich region of zyxin. Zyxin can generate new actin structures in a VASP-dependent manner, independently of the Arp2/3 complex that cooperates with members of the Wiskott-Aldrich syndrome family of proteins (WASP) to nucleate actin filaments [114, 116]. Zyxin is a VASP-dependent actin polymerization machine in cells [117], and Han et al. showed zyxin binds to the C-terminal domain of protease-activated receptor 1 (PAR-1) [114]. Upon disruption of PAR-1-zyxin interaction, thrombin-induced formation of actin stress fibers was inhibited further supporting the hypothesis that zyxin functions as a signal transducer in PAR-1 signaling. In contrast, downregulation of zyxin did not affect thrombin-induced activation of RhoA or Gi, Gq and G12/13 heterotrimeric G proteins, implicating a novel signaling pathway regulated by PAR-1 that is not mediated by G proteins. Depletion of zyxin using siRNA inhibited thrombin-induced actin stress fiber formation and serum response element (SRE)-dependent gene transcription. In addition, depletion of zyxin resulted in delay of endothelial barrier restoration after thrombin treatment. In 2017, Han et al. reported that downregulation of zyxin in HUVECs with shRNA inhibits cAMP-dependent secretion of vWF [116]. In zyxin shRNA-expressing cells, formation of the actin framework around exocytic WPBs was scarce. Moreover, phosphorylation of zyxin at serines 142 and 143 (S142/S143) is critical for vWF secretion since the zyxin mutant could not rescue the defect in zyxin shRNA-treated cells [116]. They showed that a protein kinase A (PKA)-specific inhibitor blocked zyxin phosphorylation at S142/S143 and concluded that zyxin acts downstream of PKA [116]. Han et al. thus proposed a novel model for cytoskeleton reorganization around WPBs undergoing exocytosis. Upon epinephrine stimulation, pre-existing filaments are reorganized to form actin frameworks

around exocytotic granules, limiting granule movement and promoting their localization in close proximity to the plasma membrane [116]. Then, actin monomers are recruited from the cytosol to form coat structures around granules within actin frameworks that promote fusion [116]. It was postulated that ECs use this synergistic strategy for effective and precise exocytosis. Under their experimental conditions, zyxin downregulation with shRNA had no effect on vWF release upon thrombin or histamine stimulation, whereas these mice exhibited impaired epinephrine-stimulated vWF release, prolonged bleeding time and thrombosis. Live cell super-resolution microscopy allowed visualization of zyxin-dependent reorganization of pre-existing actin filaments around WPBs before fusion. Using the total internal reflection fluorescence structured illumination microscopy (TIRF-SIM) technique, it was possible to achieve simultaneous visualization of the dynamics of fine cortical actin filaments and the behavior of the exocytotic granule in close proximity of the plasma membrane. Zyxin promotes the recruitment of the actin regulatory protein α -actinin; α -actinin is an actin crosslinking protein [114]. To prove the co-localization of zyxin with its interacting partners, they co-expressed zyxin construct tagged with mCherry for fluorescence microscopy detection (zyxin-mCherry) and Lifeact tagged with green fluorescence protein (GFP-Lifeact) (Lifeact is an actin binding peptide used in microscopy to monitor the behavior of actin filaments). Interestingly, the assembly of the pre-existing filaments started when WPBs were still tubular, so the formation of the actin framework appeared as a pre-fusion event by TIRF-SIM. Alexa Fluor 647-G actin incorporation assay indicated that pre-existing actin filaments reorganize to form the actin framework around the tubular WPBs, and G-actin was also recruited to form the actin coat structure in proximity to WPBs fused to the membrane and connected with the actin frameworks. Once WPBs became spherical and fluorescently-labeled, vWF was expelled and fluorescence intensity declined in the expelled area. The authors explain that the exocytotic events shown by variable-angle TIRF are mediated by the contraction of the actin coat which squeezes out WPB contents, followed by retraction of the depleted WPBs in the cytoplasm [116].

3. Dynamics of vWF reactivity in acute diseases

3.1. vWF as a surrogate marker of endothelial dysfunction

Association between vascular dysfunction and increased vWF levels is well established. The first study found a correlation between factor VIII antigen (another name for vWF) and kidney vascular damage in the context of glomerulonephritis in the 1970s [118]. Since this time, there have been many reports of inflammatory diseases associated with elevated vWF, that is, in acute systemic inflammation disorders such as sepsis, acute respiratory distress syndrome or systemic inflammatory response syndrome [73], T2DM [119] and TTP [120].

Upon activation of ECs, vWF acts as an acute phase reactant and correlates with serum C-reactive protein (CRP) level, another acute phase reactant [121, 122]. Other hemostatic proteins also behave in this way (i.e. factor VIII, fibrinogen and plasminogen inhibitor 1). Active vWF corresponds to the form of vWF required for platelet receptor GpIb binding [123–125].

Detection of active vWF is now possible using an assay based on a nanobody AU/vWF a11 which allows investigators to distinguish between the active and latent conformations of the vWF A1 domain [123]. Several pathological conditions are associated with a disturbed balance in vWF activation and inactivation kinetics and thereby increased levels of active vWF and thrombotic complications [126]. The same active vWF assay revealed that levels of circulating active vWF increased approximately twofold in patients with acquired and congenital TTP [123, 126]. More and more evidence indicates that vWF is a biomarker of EC activation, but there are numerous discrepancies among the various clinical studies [127, 128]. In a more recent effort to advance the use of plasma vWF as a clinical marker of vascular inflammation, Hyseni et al. measured plasma concentrations of active vWF in a cohort of 275 patients with systemic inflammatory response syndrome [45]. They reported that patients with an elevated level of active vWF on admission had a twofold higher mortality rate [45]. In contrast, despite strongly elevated vWF levels, no predictions of mortality could be obtained based on total vWF [45]. Elevated active vWF is thus now regarded as an independent biomarker of poor outcome in patients with acute lung injury [129]. Mechanical ventilation is necessary to support the critical ill, but it also exacerbates injury through mechanical stress-activated signaling pathways, therefore it is expected to affect the disease outcome [130]. Consistent with these findings, an earlier 28-day study of 50 patients van der Heijden et al. reported that high vWF levels correlated with pulmonary compliance [$V_t/(P_{plat} - PEEP)$], where V_t = tidal volume, P_{plat} = plateau pressure, $PEEP$ = positive end-expiratory pressure throughout the course of septic shock while patients were mechanically ventilated [131].

3.2. vWF/ADAMTS13 axis in vascular health and disease

In order to fulfill its functions, vWF remodels in a few distinct ways [16, 132–136]. In ECs, vWF forms tubular structures inside acidic WPBs secretory granules [137]. The switch that converts highly packed vWF tubules into ultra-large vWF strings in the blood stream is critically important but poorly understood. Recently, more insight has been gained into the mechanism of rapid transition from tightly packed vWF tubules into intraendothelial granules to vWF strings that function at physiological pH. It is likely that distal to the fused end of the WPB, alkalisation induces a rapid conformational change in the structure of vWF, which propagates causing vWF to unfurl in a concerted manner at the site of secretion, resulting in the loss of the storage conformation [132].

The highly multimeric, elongated form of vWF is not present in healthy plasma, but it is found in various pathological settings. This observation can be explained by the fact that vWF senses shear forces and remodels accordingly [134]. Atomic force micrographs have demonstrated at the single molecule level that under static conditions, vWF assumes a globular conformation, whereas, under high shear flow, vWF turns into an extended chain format [16] that forms ultra-large strings to which platelets bind to initiate clot formation at sites of vascular damage [25] and, when shear stress is above 30.000 s^{-1} , factor VIII is released from its carrier protein to provide factor VIII to the coagulation cascade [138]. We now realize that, while ultra-large MW (molecular weight) vWF is essential for the normal hemostasis, this multimeric array should not become too large because it alters the thrombotic propensity [15, 16, 133, 134, 138–147].

The last biosynthetic step required for the formation of normal vWF is the limited proteolysis of the multimeric array by the circulating metalloprotease ADAMTS13 [126, 135]. ADAMTS-13 is produced mainly by the hepatic stellate cells [148]. Secondly, the enzyme is synthesized in other cell type (ECs, podocytes, platelets and glial cells), but the physiological importance of these other sources was not yet established [149]. ADAMTS-13 plasma concentration is in the range of 0.7–1.4 $\mu\text{g/mL}$ [150]. Interestingly, ADAMTS-13 is secreted into the circulation as an active enzyme and has a plasma half-life of approximately 2–4 days [151]. The mechanism of ADAMTS-13 clearance is not completely elucidated. It has been suggested that the hepatic asialoglycoprotein receptor could be involved in ADAMTS-13 clearance [152]. It is believed that newly released multimeric forms of vWF become tethered to the EC surface through the interaction with P-selectin [153, 154] and become partially unfolded [16], allowing for ADAMTS13 cleavage of the multimeric arrays [136]. The physiological proteolytic processing of vWF by ADAMTS13 occurs between Tyr1605-Met1606 in the central A2 domain of vWF, and is facilitated by partially unfolding vWF by flow conditions in the microcirculation [124, 126, 155–162].

Too little cleavage of the newly released vWF produces a tendency for microvascular thrombotic occlusion, as in TTP [120], and too much cleavage by ADAMTS13 results in type 2 like vWD and a bleeding phenotype [38]. Cleavage is dramatically increased during systemic inflammation [45] and other prothrombotic conditions, and there is an inverse relationship between plasma levels of vWF and ADAMTS-13; under high shear stress/inflammatory conditions, the circulating vWF can acquire autoimmune resistance to proteolysis by ADAMTS-13 or ADAMTS-13 is quickly exhausted as there is more vWF to cleave than available cleaving enzyme for consumption [45]. Additionally, a decrease in the vWF-inactivating protease ADAMTS13 is dependent on the severity of the disease and organ dysfunction [163].

In mice models, vWF-deficiency abrogates the ADAMTS13-deficient prothrombotic state [164] and endotoxemia-induced thrombosis in mice [165], consistent with the observation that vWF-deficient mice subjected to the polymicrobial model of sepsis exhibit increased survival [29]. It has been recently reported that neutrophil-derived small peptides or human neutrophil peptides (HNPs) also known as α -defensins can inhibit cleavage of vWF by ADAMTS13 [166]; these peptides [167] have sequence similarities with the ADAMTS-13 spacer domain RRY motif and can bind to the cleavage site for ADAMTS-13 on the vWF A2 domain. It was shown that HNPs levels are high in TTP patients [166]. Pro-inflammatory cytokine IL-6 limits ADAMTS-13 function [168, 169], while IL-8 and TNF- α stimulate further the release of vWF from WPBs [168].

In conclusion, excessive levels of the highly prothrombotic and multimeric form of vWF and/or ADAMTS13 deficiency constitute a unifying pathologic mechanism linking inflammation to thrombosis [170].

3.3. vWF role in ischemic stroke

Stroke remains a major health concern and a leading cause of death in the adult population. As a result of intracerebral thrombosis, cerebral ischemia/reperfusion injury causes brain tissue

damage [171]. vWF promotes intracerebral thrombo-inflammatory response in the context of acute stroke [167, 172–175] and the vWF/ADAMTS13 axis was found to be involved in acute and chronic ischemic cerebrovascular events in patients [175]. vWF-deficient mice are protected from ischemic stroke [174]. It is well known that circulating vWF originates primarily from the endothelium, with a minor contribution from platelets [20]. Our current understanding of the role of different pools of vWF in the pathophysiology of acute stroke is based on mouse models [176]. EC-vWF mice are chimeric mice that express vWF only in ECs. Irradiated mice transplanted with bone marrow from vWF^{-/-} mice to repopulate myeloid cells lack vWF in platelets but express vWF in ECs. Plt-mice are chimeric vWF^{-/-} mice transplanted with normal bone marrow, and therefore have vWF in platelets [176]. The wild-type (WT), vWF^{-/-}, Plt-vWF and EC-vWF mice were subjected to 1 h ischemia followed by 23 h reperfusion and the outcome was compared [176]. The infarct volume and neurological outcome were comparable in WT and EC-vWF mice and decreased in vWF^{-/-} mice. The stroke outcome in EC-vWF mice was documented using triphenyl-tetrazolium chloride-stained serial brain sections after 1 h transient middle cerebral artery occlusion. Also, it was observed in EC-vWF mice that there was a reduced local cerebral blood flow as assessed by Doppler flowmeter and a higher rate of thrombus formation in comparison to vWF^{-/-} mice assessed by intravital microscopy. Stroke, infarction, vascular spasm and iatrogenic thrombotic events cause ischemia, which has a dramatic impact on vital organs. Post-ischemic reperfusion changes the pH, and extracellular alkalization stimulates the physiological mechanisms that directly promote EC activation and can double vWF release in a [Ca²⁺]-dependent manner [171]. Delaying the pH recovery with a Na⁺/H⁺ exchange subtype I pump inhibitor (applied in the perfusion media) [171] might prove to be a promising new way of inhibiting EC activation upon reperfusion injury. Taken together, EC-vWF seems to be the primary determinant of vWF-dependent ischemic stroke [171, 176], while platelet vWF plays a minor role [20, 176]. The ability to control vWF secretion from dysfunctional endothelium might be a valuable therapeutic target in stroke prone patients.

3.4. vWF role in sepsis

Apart from its primary role in hemostasis, vWF has a dual role in the pathophysiology of severe sepsis. On the one hand, vWF is involved in host-defense with a possible initial protective role in preventing complement activation [121]. In addition to its classical role in platelet binding, the vWF A1 domain also plays a role in vWF-dependent inflammatory responses. vWF and platelets promote leukocyte diapedesis, downstream of leukocyte tethering, rolling and adhesion in a mouse model of thioglycollate-induced peritonitis [177]. Polymorphonuclear leukocytes seem to directly interact with vWF via P-selectin glycoprotein ligand-1 and β 2-integrins [178] As shown in mice in which inflammation was provoked by two different methods, [27] vWF-regulated leukocyte recruitment can be blocked by administration of a blocking anti-vWF llama nanobody, KB-VWF-006, which has picomolar affinity for the vWF A1 domain [27].

On the other hand, sepsis is not only a systemic inflammatory condition, it is also a state of dysfunctional endothelium and coagulation. One of the early on signs of systemic endothelial

activation is sepsis-induced elevation of plasma vWF levels [121, 129, 163, 179]. Sepsis can promote DIC, which is common in critically ill patients. When unfolded, highly multimeric sepsis-induced vWF interacts with platelets [26] and generates small clots in the microvasculature [45, 124]. Microvascular thrombosis appears to be a major pathological mechanism in sepsis pathology resulting in multi-organ dysfunction syndrome (MODS) [124]. Hence, sepsis-induced vWF secretion in excess in DIC and MODS are inevitably linked. In addition, sepsis-induced ultra-large high molecular weight multimeric vWF permits complement activation, [121] leading to a positive feedback cycle of inflammation and thrombosis.

In murine model of sepsis, mice subjected to cecal ligation and puncture (CLP) have increased circulating levels of vWF that promote a procoagulant phenotype and poor outcome [29]. vWF-deficient mice subjected to the CLP model of sepsis exhibit increased survival [180]. For the purpose of testing whether Gα12 modulates vWF secretion in sepsis pathological setting, we subjected Gα12^{-/-} mice and WT control mice to the CLP model of polymicrobial sepsis and monitored survival [181]. Our preliminary results indicate that 80% of the Gα12^{-/-} mice survived, while all WT mice succumbed in the first 96 h [182]. To further determine whether decreased vWF was responsible for the protective effect of G12 deficiency, we restored circulating vWF level in Gα12^{-/-} mice, by intravenous administration of purified vWF [10], to the level observed in WT mice. This procedure resulted in the loss of the protective effect of G12 deletion [182]. Furthermore, we employed a pharmacological G12 inhibition approach to prove G12 involvement in sepsis-induced vWF secretion [183]. We hypothesized that a synthetic peptide derived from Gα12 would inhibit α-SNAP-dependent WPB priming and fusion with the plasma membrane [89, 182], thereby inhibiting vWF secretion from activated ECs, and reducing the risk of thrombotic microangiopathy during sepsis [182].

3.5. vWF role in other inflammatory diseases

Transient elevations of plasma vWF level were also observed after epinephrine infusion [184], and chronic elevation of circulating vWF (2–3-fold) was reported in hyperthyroidism, which is thought to be due to β-adrenergic receptor stimulation. In contrast, hypothyroidism is associated with a 15% decrease in vWF level, although this is reversible with appropriate treatment [185].

vWF secretion from ECs might play a role in sickle cell disease pathogenesis [186, 187]. Erythrocytes bind specifically to vWF [186] and stasis, or the reduction of blood flow and shear, promotes binding of erythrocytes to vWF which form fibrin-rich regions in venous thrombi [186].

4. vWF-related antithrombotic treatment strategies

Despite recent major advances in the vascular biology of thrombosis, we face unmet treatment needs which warrant search for novel antithrombotic medication. Inhibition of vWF secretion constitutes an attractive therapeutic strategy to counteract thrombus initiation and propagation as the plasma concentration of vWF increases in high risk populations and predicts cardiovascular disease outcome. In this section, we review the drugs with the potential of

vWF antagonism, illustrating how they might become an option to overcome current limitations of antithrombotic therapy.

Most of the studies involving anti-vWF antibodies were conducted in animal models. Monoclonal antibodies have been widely tested in murine, monkey and rabbit models of cardiovascular disease, including coronary thrombosis, stroke and in-stent stenosis: GPG-290, 6B4-Fab, AJW200, 82D6A6 and SZ-123 [188, 189]. These are all agents with antithrombotic and anti-inflammatory effects which do not prolong bleeding time and do not provoke thrombocytopenia. However, to date, none of these have made it into clinical trials. Hillgruber and colleagues recently published a study that found a massive accumulation of vWF in skin biopsies of patients suffering from leukocytoclastic vasculitis, an immune complex (IC)-mediated vasculitis (ICV) frequently encountered in dermatology that is caused by IC precipitation in the vessel wall followed by recruitment of neutrophils [28]. These results were confirmed in a murine model of vasculitis [28], and importantly, they identified ICs as possible vWF secretagogues. Of note, polyclonal anti-vWF blocking antibodies had an anti-inflammatory effect, reducing leukocyte recruitment and edema formation in a murine model of vascular inflammation [28]. Although the physiological implications of elevated vWF levels (increasing up to 400% of the normal level in various conditions [190, 191]) require additional investigation, it is currently believed that plasma vWF level is a surrogate marker of increased risk of vascular complications in septic patients and in those with vascular disease, which makes vWF antagonism a promising therapeutic target in thrombosis and inflammation.

Aptamers and nanobodies have been demonstrated to directly interfere with the vWF pathway, suggesting their powerful antithrombotic properties and acceptable level of safety may prove to be useful strategies. Aptamers are oligonucleotides that have similar characteristics with monoclonal antibodies and with small molecules. ARC1779 is an aptamer that binds to the A1 domain of vWF, blocking its interaction with GpIb and thereby inhibiting vWF-dependent platelet function. Clinical trials with ARC1779 were conducted and completed, but until truly meaningful clinical endpoints can be obtained that indicate effective inhibition of thrombosis without increasing bleeding, the utility of aptamers will remain unclear. Before the clinical trial was prematurely closed by the sponsor, the clinical and laboratory data after 14 days of dosing in nine patients with TTP aged 18–75 were very promising, and the authors optimistically proposed the study continuation (www.clinicaltrials.com; [192]) suggesting further development may be in order.

Nanobodies have the advantage of being highly specific therapeutic agents. Caplacizumab (ALX-0081) is an anti-vWF humanized nanobody that selectively targets vWF A1 domain, locking platelets launching on VWF A1 domain via interaction with their receptor GPIb. TITAN phase II trial concluded that caplacizumab administration in 75 patients with acquired TTP lead to a rapid resolution of the acute TTP episode, but it was adversely seconded by mild bleeding when compared to placebo group [193–195].

Unlike the vWF aptamer or antibodies, a pharmacological inhibitor of vWF secretion would have the advantage of acting from the inside of ECs, thereby limiting the amount of vWF available to support binding of platelets and thrombus formation. Studies thus far from our group suggest inhibition of $G\alpha_{12}$ -dependent activation of vWF secretion from WPBs may in

fact be one such option [89, 90, 181, 182, 196]. Ongoing studies are demonstrating that inhibition of vWF secretion from ECs, using a novel peptide inhibitor to block $G\alpha_{12}/\alpha$ -SNAP-dependent WPB exocytosis [89], reduces DIC and mortality in septic mice [89, 90, 182, 183, 196].

5. Conclusions

The development of antithrombotic drugs continues to be an active area of research. As there is not a straightforward relationship between therapeutic intervention and improvement of endothelial function, but rather a complicated interrelation between multiple subcellular “targets”, research has focused on understanding the underlying mechanisms leading to vWF elevation in the circulation. Development of novel non-invasive diagnostic methods that facilitate early detection of endothelial damage and dysfunction and expand our knowledge of the etiology of cardiovascular disease are aggressively being pursued. In this overview, we present currently available literature concerning the contribution of endothelial cell activation/dysfunction to the increase in the level of plasma vWF in the context of inflammatory cardiovascular disease. While differential diagnosis of infectious and non-infectious organ damage associated with high vWF levels is not possible using vWF as a marker, elevated levels of vWF correlate very well with organ failure and poor survival and thus encourage further pursuit of this line of research toward novel drug therapy.

Author details

Luiza Rusu* and Richard D. Minshall

*Address all correspondence to: luizarusu123@gmail.com

Ohio State University, University of Illinois at Chicago, USA

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