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Von Willebrand Factor remodeling during exocytosis from vascular endothelial cells

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Abstract

Background: In vascular endothelial cells high molecular weight multimers of Von Willebrand factor (VWF) are folded into tubular structures for storage in Weibel-Palade bodies (WPBs). Upon stimulation, VWF is secreted and forms strings to induce primary hemostasis. The structural changes comprising the transition of stored tubular VWF into secreted unfurled VWF strings are still unresolved even though they are vital for normal hemostasis. The secretory pod is a novel structure that we previously described in endothelial cells. It is formed upon stimulation and has been postulated to function as a VWF release site. In this study we investigated the actual formation of secretory pods and the subsequent remodeling of VWF into strings. *Methods:* Human Umbilical Vein Endothelial Cells (HUVEC) were stimulated and studied using various imaging techniques such as live-cell imaging and correlative light and electron microscopy. *Results:* We found by live-cell imaging that secretory pods are formed by coalescence of multiple WPBs without involvement of other large structures. Secreted VWF expelled from secretory pods was found to adopt a globular conformation. We visualized that VWF strings derive from those globular masses of VWF. Flow experiments showed that upon secretion the globular masses of VWF move to the edge of the cell where they anchor and generate VWF strings. *Conclusion:* Upon secretion VWF adopts a globular conformation which remodels into strings after translocation and anchoring at the edge of the cell. This finding reveals new pathophysiological mechanisms that could be affected in patients suffering from Von Willebrand disease.

7.1 Introduction

Vascular endothelial cells (ECs) contain specialized storage organelles, the Weibel-Palade bodies (WPBs).^{1,2} The main constituent of WPBs and a prerequisite for their formation is von Willebrand factor (VWF).^{3,4} Multimers of VWF are formed at the Trans Golgi network and are stored as compact helical tubules⁵⁻⁷, together with proteins such as P-selectin, IL-8 and angiopoietin-2, in WPBs.⁸ In response to agonists such as thrombin and epinephrine ECs are signaled to induce WPB exocytosis.⁹ Once VWF is released from ECs, long strings are formed which capture platelets to initiate primary hemostasis.¹⁰ Deficiencies in the exocytotic mechanism could result in impaired hemostasis as seen in patients with Von Willebrand disease (VWD).¹¹ It has been proposed that VWF tubules in WPBs are spring-loaded^{10,12} to provide a rapid and efficient mechanism to unfurl VWF multimers upon exocytosis. VWF strings would then directly emerge from single WPBs. Recently we have identified a novel structure involved in VWF secretion, which we termed secretory pods.¹³ Secretory pods are formed upon stimulation and are hypothesized to function as a VWF release site following multigranular WPB exocytosis. In this secretion mode multiple WPBs pool their content together in secretory pods, before fusion with the plasma membrane. The VWF tubules of WPBs are unfurled in secretory pods¹³ suggesting that the tubular packaging of VWF in WPBs is not essential for secretion and VWF string formation. For in depth characterization of this mode of secretion we used various sophisticated imaging techniques to explore the origin of secretory pods and the remodeling of VWF into strings upon secretion.

7.2 Methods

Cell culture and transfection

Human Umbilical Vein Endothelial Cells (HUVEC) (Lonza, Walkersville, MD) were cultured for 8 days.¹² For transfection we used enhanced green fluorescent protein (EGFP) tagged VWF propeptide (propeptide-EGFP) containing the D1-D2 domains of full-length VWF (from Dr. Carter, MRC National Institute for Medical Research, London, UK).¹⁴ Transfections were performed using Nucleofection (Lonza) or jetPEI™-HUVEC (Polyplus Transfection, Illkirch, France). Nucleofection was performed using the HUVEC Nucleofector® Kit (Lonza). For each reaction we used 2 µg DNA, 1×10^6 cells and program A-034. Cells were grown for 48 hours and medium was refreshed after 24 hours. The JetPEI™HUVEC

reagents (Polyplus Transfection) and DNA were incubated with each other in a 2:1 ratio according to the manufacturer's protocol. Complexes were incubated with the cells for 2 hours. Medium was replaced and cells were kept in culture for 48 hours before use in experiments. No differences between the two transfection methods were observed.

Regulated WPB exocytosis

To induce WPB exocytosis, cells were stimulated with 80 nM phorbol 12-myristate 13-acetate (PMA).¹³ For immunolabeling, cells were fixed in 3% paraformaldehyde (PFA) in 0.1 M phosphate buffer pH 7.4 for 30 minutes at 4 °C. For Correlative Light and Electron Microscopy (CLEM) 3% PFA 1% glutaraldehyde (GA) in 0.1 M phosphate buffer pH 7.4 was used. Fixative was replaced for 0.1% PFA in 0.1 M phosphate buffer pH 7.4 for storage at 4 °C. Cells stimulated under flow were grown on μ -slide I 0.8 Luer ibidiTreat flow chambers (Ibidi, Munchen, Germany). Stimulation medium containing 80 nM PMA was perfused over the cells at 2.5 dyne/cm² using the Ibidi pump system (Ibidi). Cells were fixed afterwards in 3% PFA.

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Immunofluorescence microscopy

After fixation, cells were rinsed in 50 mM Tris-HCl pH 7.4. Unless indicated otherwise, cells were permeabilized and blocked in PBS 5% Normal Goat Serum (DAKO, Glostrup, Denmark), 0.1% Triton X-100 which was followed by incubations with primary and secondary antibodies. Cells washed with PBS were mounted on glass slides in Aquapolymount (Omnilab, Warrington, PA). Primary antibodies were fluorescein isothiocyanate (FITC) conjugated sheep anti-VWF antibody (Abcam, Cambridge, UK), rabbit anti-VWF antiserum (DAKO) and mouse monoclonal anti-CD62P (P-selectin) antibody [AK-6] (Abcam). Secondary antibodies were goat anti-rabbit or goat anti-mouse antibodies conjugated with Alexa 488 or Alexa 568 (Invitrogen Molecular Probes, Eugene, OR). Cells were imaged using a Leica SL with a 63 $\times$ /1.25 NA oil objective or a Leica SP5 (Leica Microsystems, Wetzlar, Germany) with 63 $\times$ /1.40 NA oil objective confocal laser scanning microscope. Minor linear adjustments of dynamic range were carried out in Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA).

Live-cell imaging and flow experiments

Secretory pod formation was imaged in a propeptide-EGFP expressing cell upon adding the PMA solution. The PMA solution was prepared in Endothelial Growth medium-2 (EGM-2 BulletKit, Lonza) as it appeared to preserve the fluorescence better than regular incubation medium used for stimulation. Control cells were incubated with diluted vehicle.

For flow experiments, HUVECs were grown in flow chambers (Ibidi) and a region of interest was selected. EGM-2 medium supplemented with PMA and 1:30000 diluted anti-VWF Alexa 568 antibodies¹³ was perfused over the cells at 2.5 dyne/cm² using the Ibidi pump system (Ibidi). An APEX™ Alexa Fluor 568 Antibody Labeling Kit (Invitrogen Molecular Probes) was used to conjugate polyclonal anti-VWF antibodies (DAKO) to Alexa 568.

Time-lapse imaging was performed with a climate controlled (37 °C and 5% CO₂) Leica AF6000LX equipped with a 63×/1.30 NA glycerin objective and a high speed CCD camera (C9100-02-COM7, Hamamatsu Photonics, Hamamatsu, Japan). Z-stacks were recorded at 10- or 15-second interval for 30 minutes. Maximum intensity projections of z-stacks were made and the dynamic range was adjusted (linear adjustments). Image stacks in Figure 7.6C were processed using 3D blind deconvolution of the LASAF software package of Leica.

Immunogold labeling and Scanning Electron Microscopy

Immunolabeling of secreted VWF was performed similarly as for immunofluorescence, however without permeabilization. Cells were incubated with polyclonal anti-VWF antibodies (DAKO) and with 15 nm Protein-A gold particles. After labeling, cells were fixed in 2% GA in 0.1 M sodium-cacodylatebuffer (pH 7.4), dehydrated and critical point dried.¹³ Coverslips with cells were then mounted on stubs, carbon coated using an Emitech K950 evaporator (Ashford, UK) and viewed with a JEOLJSM-6700F field emission SEM at 15 kV (Jeol, Tokyo, Japan). The secondary electron image detector was used to image morphology; the back-scatter detector was used to visualize gold label.

Correlative light and electron microscopy

HUVECs were grown on grid engraved glass bottom dishes (MatTek Corporation, Ashland, MA). After stimulation and fixation cells were labeled for VWF as described for immunofluorescence microscopy. Cells were kept in fixative to

enable subsequent sample preparation for TEM after imaging fluorescence with a Leica SP5 confocal laser scanning microscope. Embedding and sectioning were performed as described.¹³ Correlated fluorescence and electron micrographs were scaled, flipped, rotated, and overlaid in Adobe Photoshop CS3 using the nuclei in the area of interest to align the two images. EM was performed using a Tecnai 12 TEM at 120 kV (FEI Company, Eindhoven, The Netherlands) equipped with a 4kx4k CCD camera (Model Eagle, FEI Company). For serial electron tomography 10 nm protein-A gold was used as fiducials. Double tilt series from -60° to $+60^\circ$ were collected at 1° interval using Serial EM.¹⁵ 3D reconstructions were generated and combined using the IMOD package.¹⁶ For segmentation using the AMIRA Visualization Package (Visage Imaging GmbH, Berlin, Germany), tomograms were binned 2 times and denoised by nonlinear anisotropic diffusion using the IMOD package.

7.3 Results

Previously, we studied secretory pods by adding VWF specific antibodies to the stimulation medium to keep secreted VWF at the site of exocytosis and to prevent VWF string formation, as we were especially interested in studying the site of exocytosis.¹³ To study the nature of secretory pods and the remodeling of VWF upon secretion in more physiological conditions we have now investigated stimulated HUVECs without perturbing the VWF secretion with high doses of anti-VWF antibodies.

Secretory Pods are formed by multiple WPBs on endothelial stimulation

HUVECs stimulated in the absence of antibodies were stained after fixation with fluorescent antibodies to reveal endogenous VWF. To discriminate between extra- and intracellular VWF we labeled extracellular VWF first before permeabilizing the cells for intracellular VWF staining (Figure 7.1A). Extracellular VWF labeling revealed VWF strings which were often connected to bright VWF positive dots (Figure 7.1A) suggesting that strings are formed from VWF dots. Intracellular VWF labeling revealed WPBs and also large VWF containing dots, the secretory pods (Figure 7.1A). Some secretory pods colocalized with extracellular VWF suggesting VWF release sites. Also a strong association was observed between WPBs and secretory pods suggesting that secretory pods are formed by WPBs. In Fig-

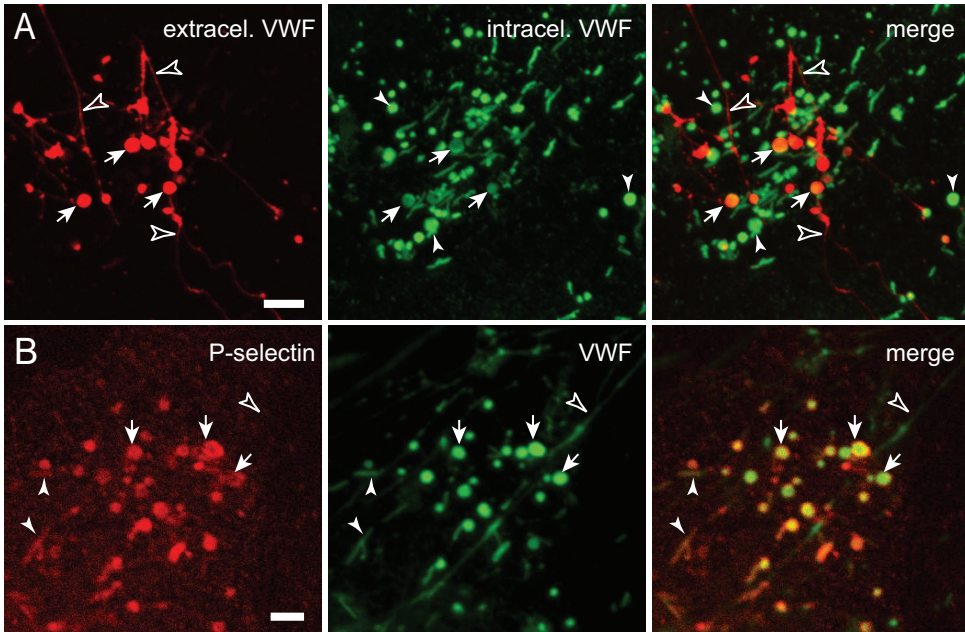


Figure 7.1. Secretory pods in PMA stimulated endothelial cells. (A) Extracellular VWF (left panel, red) labeled after fixation without permeabilization. VWF strings (open arrowheads) are connected to VWF dots (arrows). After permeabilization intracellular VWF (middle panel, green) was stained showing WPBs and secretory pods (closed arrowheads). Scale bar, 5 μm . (B) Immunolabeling with anti-P-selectin (left panel, red) and anti-VWF (middle panel, green) in permeabilized cells showed P-selectin labeled WPBs (closed arrowheads) and secretory pods (arrow). Scale bar, 2 μm .

ure 7.1B we show that P-selectin, a WPB compound, is also found on secretory pods.

Previously we observed in electron tomograms WPB fusion to a secretory pod.¹³ However the onset of the actual secretory pod formation still remained to be elucidated. To examine if WPBs are capable of homotypic fusion to form secretory pods live-cell imaging experiments were performed. HUVECs were transfected with propeptide-EGFP to label the WPBs. This EGFP construct codes for the propeptide, domains D1-D2 of full-length VWF, which is normally cleaved from VWF and is non-covalently incorporated in the helical VWF tubules during WPBs formation. Although secretory pod formation is not exclusive for PMA stimulation¹³, we used PMA as it acts much slower on the cells than other secretagogues facilitating the imaging of fusion events. Exocytotic events were observed as the disappearance of the fluorescence when propeptide-EGFP diffuses away

upon release.¹⁴ After adding the secretagogue we observed that WPBs rounded up into small propeptide-EGFP containing dots undergoing single WPB exocytosis (Figure 7.2A-Ai). We also observed (n=12 distributed over 6 experiments) the formation of secretory pods in which WPBs fused with each other (Figure 7.2B-Bi and C-Ci). Coalescing WPBs sometimes appeared to fuse directly into one structure (Figure 7.2Bi and supplemental Video 1) while other WPBs became round prior to fusion (Figure 7.2Ci and supplemental Video 2). Based on the calculated volumes of the WPBs coalescing into a secretory pod we could conclude that the total volume of WPBs contributing to a secretory pod corresponded to the calculated volume of the formed secretory pod, assuming the secretory pod to be spherical. This suggests that no other large structures are involved in secretory pod formation and that the organelle is completely formed by fusing WPBs. Transfected cells (n=6) with secretory pods contained 1 to 3 secretory pods (mean 1.7 ± 0.8) which were created by 2 to 4 WPBs and had diameters from 0.65 to 1.5 μm (mean $1.1 \mu\text{m} \pm 0.3$). After WPB coalescence full collapse of secretory pods was observed. Control cells did not show exocytotic events or stress reactions, nor obvious differences in photo bleaching during imaging (supplemental Figure S1).

VWF released from secretory pods is not directly remodeled into strings

It is unknown how VWF tubules in WPBs give rise to VWF strings upon exocytosis from ECs. Previously we showed that VWF in secretory pods is unfurled and adopts a dense globular conformation upon secretion when studied in the presence of anti-VWF antibodies thereby inhibiting VWF string formation.¹³ To investigate physiological VWF remodeling into strings CLEM was performed on stimulated ECs, combining confocal laser scanning microscopy with conventional TEM. Stimulated cells were fixed and incubated with fluorescently labeled anti-VWF antibodies without permeabilizing the cells, staining secreted extracellular VWF only. We also performed experiments in which intracellular VWF was stained as well. The cells were then permeabilized after labeling extracellular VWF. By confocal laser scanning microscopy, we imaged areas showing fluorescently labeled extracellular VWF dots that were connected to VWF strings (Figure 7.3A and box in Figure 7.4A). We chose these areas as VWF double labeling for intra- and extracellular VWF (Figure 7.1A) showed that these areas often colocalize to intracellular structures and suggested sites of VWF remodeling into strings. Correlating the extracellular fluorescent VWF dots to TEM sections (Figure 7.3B and Figure 7.4B) revealed globular, electron dense masses of secreted VWF (n=49, dis-

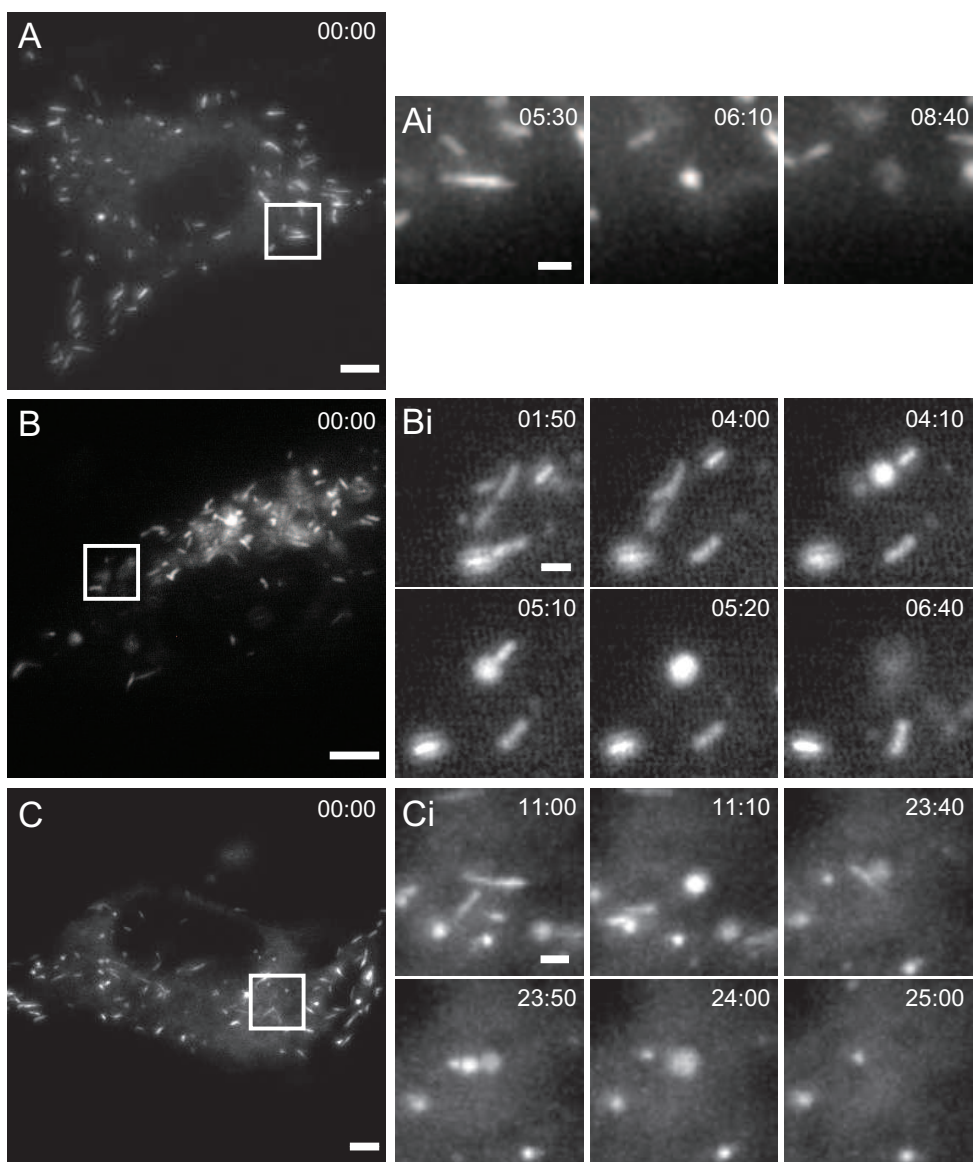


Figure 7.2. Live-cell imaging of secretory pod formation. HUVECs transfected with propeptide-Enhanced green fluorescent protein (EGFP) were stimulated with phorbol-12-myristate-13-acetate (PMA). (A-C) Overviews of imaged cells at time point 0. Boxes indicate the areas of exocytosis events depicted in panels Ai-Ci. (Ai) Snapshots of time-lapse showing exocytosis of a single WPB. (Bi) Coalescence of 4 WPBs into one secretory pod. The fusion of WPBs seems to occur directly. (Ci) Secretory pod formation by 2 WPBs. Both WPBs round up before they coalesce. (A-C) Scale bar, 5 μm . (Ai-Ci) Scale bar, 1 μm . Time after adding stimulation medium is depicted in minutes: seconds. See also supplemental Video 1 and Video 2.

tributed over 12 experiments) (Figure 7.3Ciii and Figure 7.4Ci-iii). We observed that VWF strings associated with globular VWF masses ($n=23$, distributed over 12 experiments; Figure 7.4Ci-iii) suggesting that VWF strings emerge from an extracellular globular mass of VWF. Examination of fluorescently labeled VWF dots in consecutive TEM sections showed that extracellular globular masses of VWF originated from intracellular secretory pods ($n=5$, distributed over 3 experiments, Figure 7.3C and Figure 7.4C, supplemental Videos 3 and 4). Some of the secretory pods had released most of their VWF as little electron dense material was found within the secretory pod (Figure 7.3C). Globular VWF masses were found to be located above the secretory pod (Figure 7.3C-Ciii) and were sometimes observed to exit the secretory pod (Figure 7.4Civ). Furthermore, connections between secreted VWF and secretory pod could be observed (Figure 7.3Cii and D) which may represent VWF anchoring sites. Combining tomograms from five serial sections visualized VWF emerging from the secretory pods (supplemental Video 3). Segmentation of the serial tomograms illustrates the 3D organization of the exocytosis event, highlighting the secreted emanating globular VWF mass located outside the cell (Figure 7.3E and supplemental Video 3).

Fluorescent dots of extracellular labeled VWF were also correlated to secretory pods containing lacy material (Figure 7.4Civ-v) which appeared less electron dense and less globular than the globular mass described above. This lacy material might represent VWF before secretion. To determine whether the lacy material indeed represents intracellular VWF inside secretory pods, we combined CLEM with an extra- and intracellular labeling for VWF on stimulated cells (Figure 7.4). By confocal laser scanning microscopy, we imaged areas showing both extracellular fluorescently labeled VWF dots that were connected to VWF strings and intracellular dots representing intracellular non-fused secretory pods (Figure 7.4A). Correlating fluorescently labeled intracellular secretory pods to TEM sections (Figure 7.4B) revealed that non-fused pods ($n=7$, distributed over 2 experiments) solely contained lacy VWF (Figure 7.4D). This suggests VWF was first conveyed to secretory pods as lacy material and then secreted as a globular mass before being remodeled into strings.

To support the CLEM data, we verified if we could detect globular VWF masses by SEM. Stimulated HUVECs were fixed and labeled using anti-VWF antibodies and 15 nm proteinA-gold particles. Similar to the TEM data we observed VWF masses which were connected to strings and decorated with VWF-gold label (Figure 7.5).

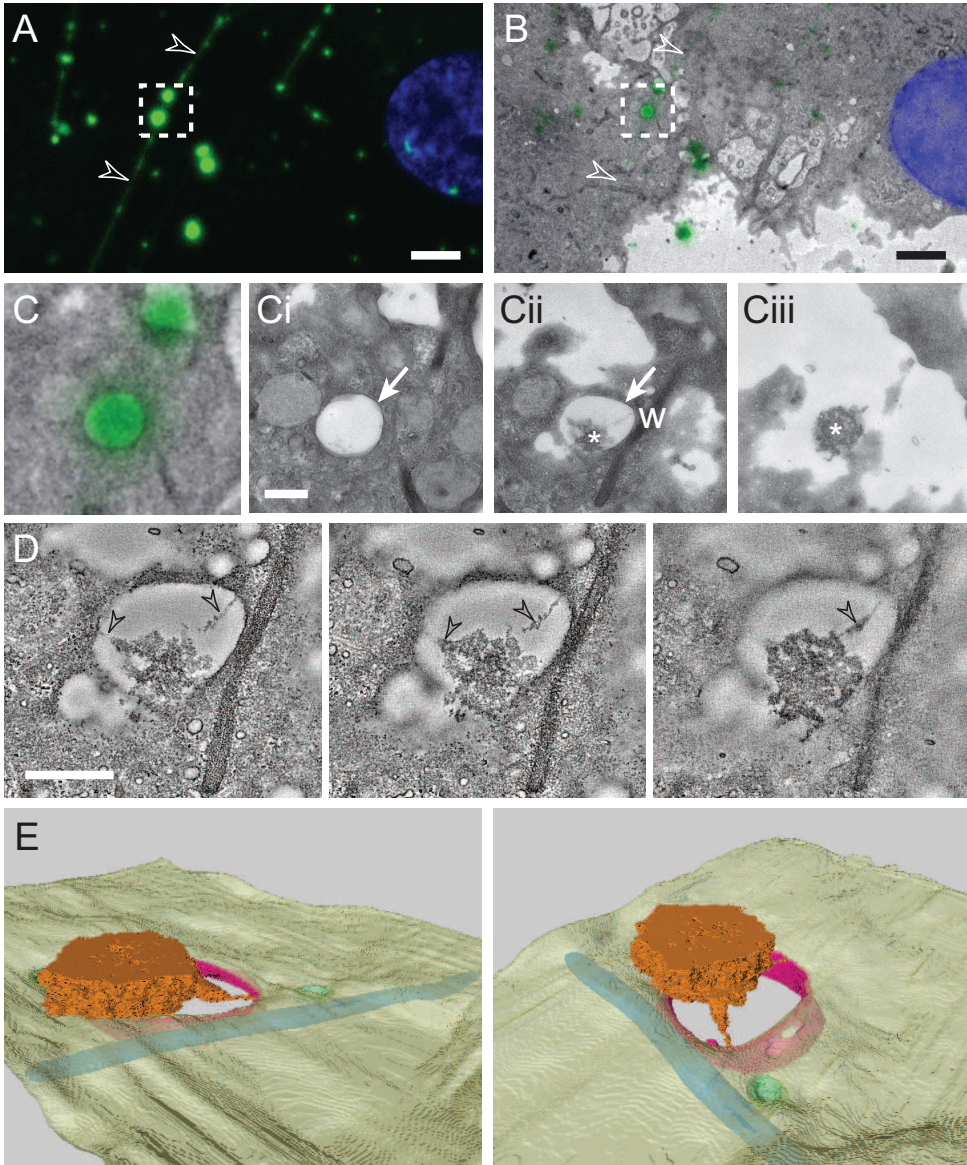


Figure 7.3. Correlative Light and Electron Microscopy on secreted VWF. HUVECs stimulated with PMA were fixed and fluorescently labeled for CLEM. (A) Fluorescence image of extracellular VWF dots (green) connected to VWF strings (arrowheads) and a nuclear stain (blue) of 4',6-Diamidino-2-phenylindole, dihydrochloride (DAPI). Cells were not permeabilized during staining. Scale bar, 5 μ m. (B) Overlay of the fluorescence image shown in panel A and the electron micrograph of a corresponding TEM section. (C) High magnification of the boxed area in panel B. (Ci-iii) Electron micrographs of 3 consecutive sections of the correlating area. (Ci) Secretory pod containing a little dense material representing secreted VWF. (Cii) More dense VWF material

Figure 7.3. (cont.) is observed in the secretory pod (asterisk) and a docked WPB (w) is revealed next to the secretory pod. (Ciii) Globular VWF mass outside the cell (asterisk). Scale bar, 500 nm. (D) Digital slices through the electron tomogram from the area shown in panel Cii showing connections between globular VWF and the secretory pod membrane (arrowheads). Scale bar, 500 nm (E) Segmented 3D model of VWF exocytosis from five combined serial tomograms (Orange, secreted VWF; Pink, secretory pod membrane; Blue, docked WPB; Yellow, plasma membrane; Green, clathrin coated pits). See also supplemental Video 3.

We show that in the absence of anti-VWF antibodies, VWF still adopts a globular conformation upon secretion which indicates that the globular VWF represents a physiological step in VWF remodeling upon exocytosis.

Flow induced VWF translocation and string formation upon secretion

To examine the association between secreted globular VWF masses and string formation in relation to flow, ECs were grown in flow chambers and perfused with medium containing PMA and very diluted red fluorescent anti-VWF antibodies to detect secreted VWF. The extremely low dose of anti-VWF antibodies allows VWF string formation and is still visible by fluorescence microscopy.¹³ Live-cell imaging of stimulation under static conditions revealed patches of VWF on top of the cells but no strings (Figure 7.6A). The fluorescent signal was quite faint under static conditions due to the low availability of antibodies in the medium. After 30 minutes of stimulation, fresh stimulation medium with fluorescent antibodies was perfused over the cells at 2.5 dyne/cm². Interestingly, we then observed that secreted VWF aggregated at the edge of the cell (data not shown). VWF strings were observed at these areas suggesting that VWF anchors at these sites after secretion. When stimulation was started under flow conditions a similar pattern was observed (Figure 7.6B, n=90, distributed over 6 experiments).

To determine the sequence of events in VWF secretion and string formation more thoroughly, we performed similar flow experiments on cells which transiently expressed propeptide-EGFP to label WPBs. As shown in Figure 7.6C and supplemental Video 5 we observed that upon WPB fusion a transition of labeling occurred. When the green propeptide-EGFP signal of WPBs disappeared, red dots (n=36 distributed over 6 experiments) of secreted VWF became visible which subsequently moved in the direction of the flow and formed aggregates at the edge of the cell. From the aggregates we observed emanating VWF strings

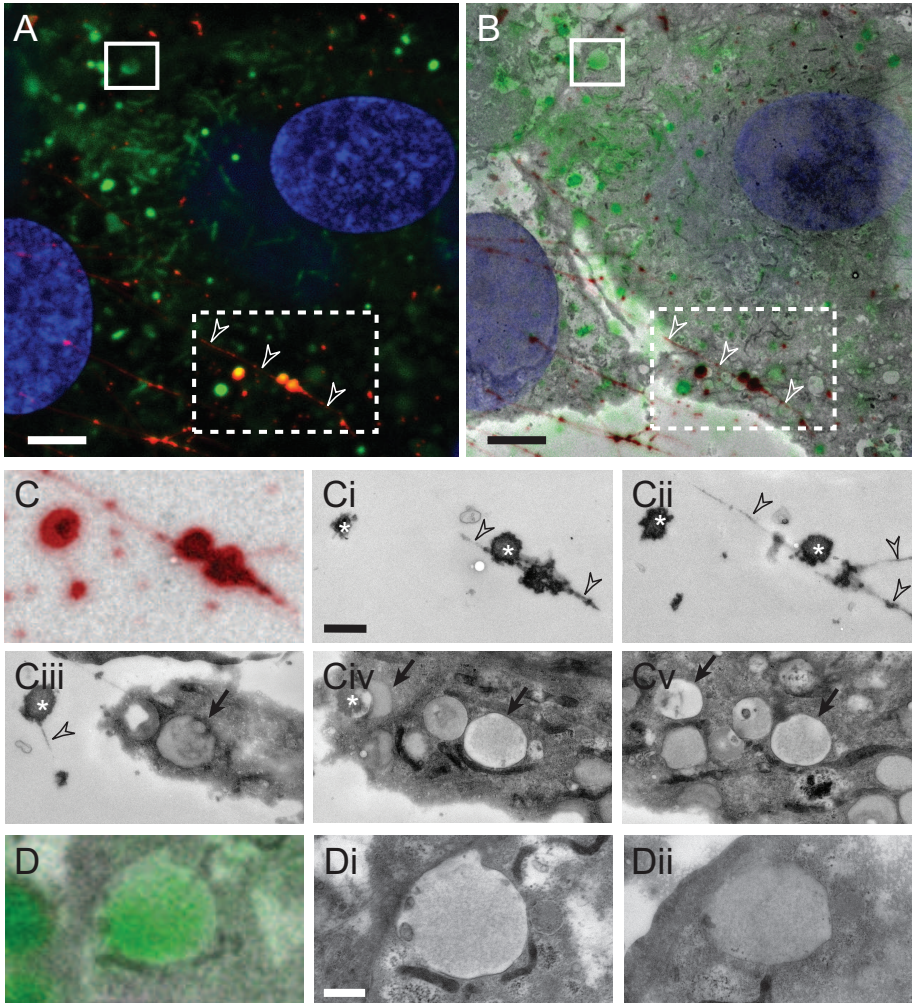


Figure 7.4. VWF remodeling upon exocytosis visualized by Correlative Light and Electron Microscopy. PMA stimulated HUVECs fixed and fluorescently labeled for CLEM. (A) Fluorescence image showing extracellular VWF dots (boxed area dashed line) connected to VWF strings (arrowheads) in red, intracellular WPBs and non-fused pods (boxed area, solid line) in green and a nuclear stain (blue) of 4',6-Diamidino-2-phenylindole, dihydrochloride (DAPI). After fixation secreted VWF was labeled first in red. Cells were then permeabilized and intracellular VWF was labeled in green. Scale bar, 5 μ m. (B) Overlay of the fluorescence image of panel A and the electron micrograph of a corresponding TEM section. (C) High magnification of the dashed lined box in panel B. (Ci-v) Electron micrographs of 5 consecutive sections of the correlating area. Ci-ii) Globular VWF masses (asterisk) connected to VWF strings (arrowheads). (Ciii) Globular VWF mass (asterisk) connected to VWF strings (arrowheads) and a secretory pod (arrow) which seemed to have secreted globular VWF. (Civ) Globular VWF (asterisk) exiting the cell and a secretory pod

Figure 7.4. (cont.) (arrow) containing lacy material. (Cv) Secretory pods (arrows) which seem to have expelled globular VWF. The right arrow shows a secretory pod (arrow) containing lacy material. (Cv) Secretory pods (arrows) which seem to have expelled globular VWF. The right arrow shows a secretory pod containing lacy material. Scale bar, 1 μm . See also supplemental Video 4. (D) High magnification of the solid line boxed area in panel B. (Di-ii) Electron micrographs of 2 consecutive EM sections. Non-fused secretory pod contains lacy material of unfurled VWF. Scale bar, 1 μm .

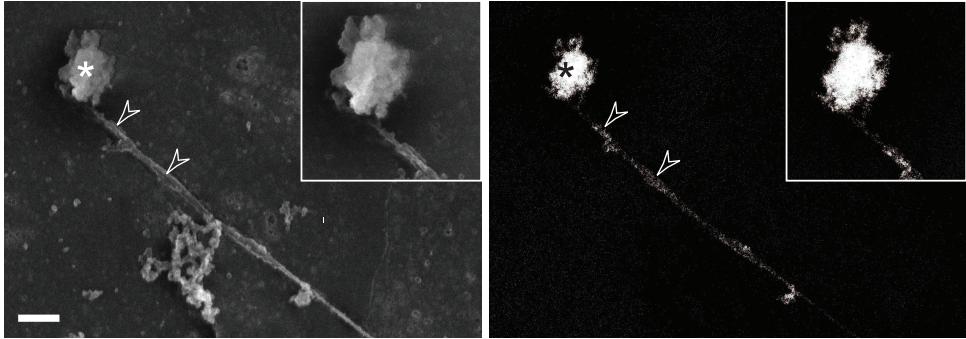


Figure 7.5. Immunogold labeling of secreted VWF revealed by Scanning Electron Microscopy. PMA challenged HUVECs labeled with anti-VWF antibodies and protein-A gold (15 nm). A secreted globular mass of VWF (asterisk) with an emerging VWF string (arrowheads, upper panel) decorated with anti-VWF gold particles (lower panel). Scale bar, 1 μm .

($n=6$ distributed over 2 experiments) indicating that strings are formed after secretion, translocation and anchoring (Figure 7.6D and supplemental Video 5).

To control for the effect of the antibodies during stimulation we stimulated cells without antibodies in the medium. These cells were fixed and stained with fluorescent anti-VWF antibodies. We observed the same pattern as observed with live-cell imaging (data not shown).

7.4 Discussion

The secretory pod was previously found to be involved in VWF secretion from ECs.¹³ Now we show three novel features of VWF exocytosis: 1. secretory pods truly originate from coalescence of multiple WPBs; 2. VWF is secreted as a globular mass of protein before remodeling into VWF strings, even when interference of anti-VWF antibodies was excluded; 3. the secreted VWF moves in the direction of flow to the edge of the cell where it anchors and generates strings.

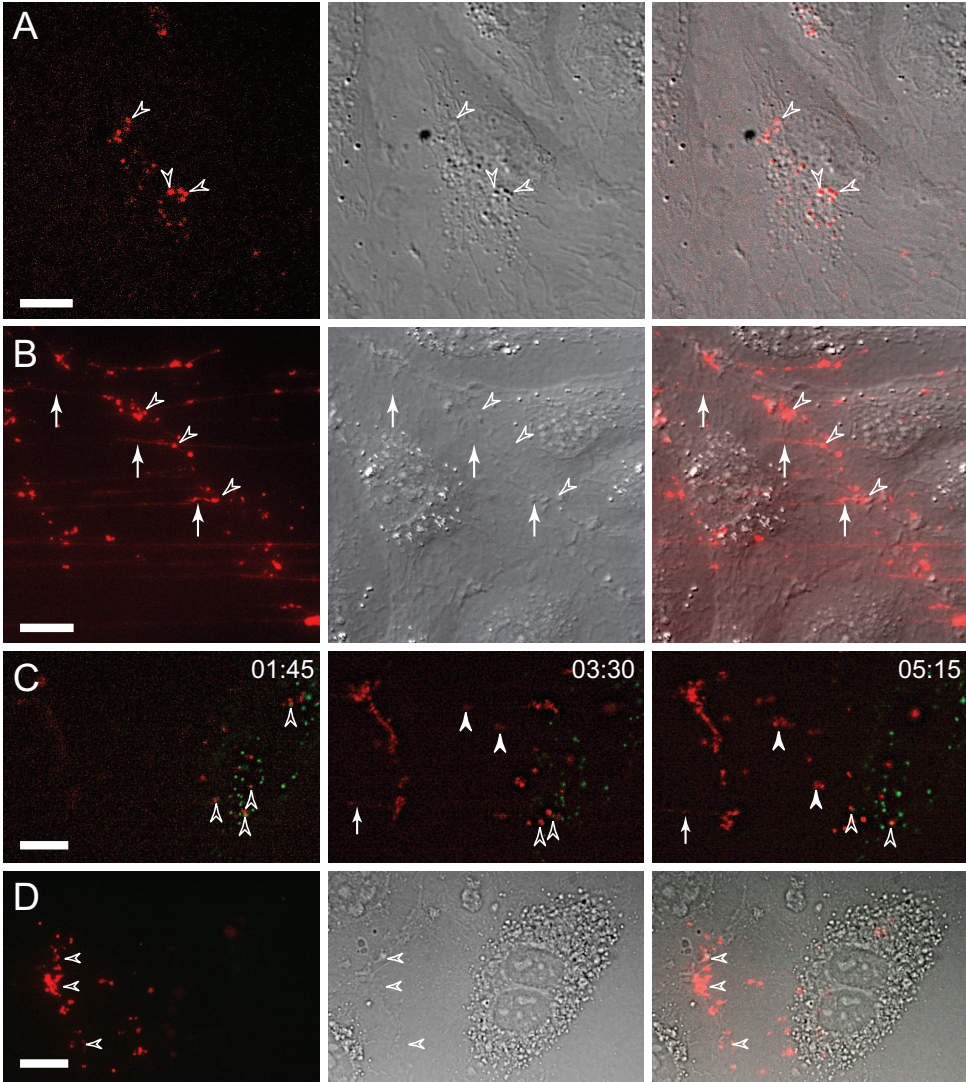


Figure 7.6. VWF string formation under flow conditions. HUVECs stimulated under static and flow conditions (2.5 dyne/cm^2). In panels B-D the flow direction is from right to left. Secreted VWF was visualized with diluted red fluorescent anti-VWF antibodies in the stimulation medium. (A) Static stimulated HUVEC showing patches of VWF (left panel, arrows) on top of the cell as shown in the differential interference contrast (DIC) image (middle panel) and merged image (right panel). (B) Snapshot from time-lapse series of HUVECs stimulated under flow. Fluorescent VWF strings (left panel, arrows) originating from globular VWF aggregates (arrowheads) at the edge of the cell as shown in the DIC image (middle panel, arrowheads) and the merged image (right panel, arrowheads). (C) Time-lapse series of propeptide-enhanced green fluorescent protein (EGFP) transfected cells showing green labeled WPBs inside the cell. Upon WPB exocytosis propeptide-EGFP diffuses away while secreted VWF becomes labeled with diluted red fluorescent

Figure 7.6. (cont.) anti-VWF antibodies (open arrowheads). Secreted VWF moves in the direction of the flow (closed arrowhead) and accumulates at the edge of the cell. From there VWF strings were observed (arrow). See also supplemental Video 5. (D) Final frame time-lapse series panel C. Fluorescent VWF labeling (left panel), DIC image (middle panel) and merge (right panel) showing aggregated VWF at the edge of the cell (arrowheads). Scale bars, 10 μm .

Imaging the formation of secretory pods showed that secretory pods are formed by homotypic fusion of multiple WPBs in which no other large organelles seem to be involved. In some cases we observed that the onset of secretory pod formation appeared very similar to lingering kiss exocytosis¹⁷ in which WPBs become round at the plasma membrane, creating a 12 nm fusion pore to release small WPB compounds such as IL-8 and the membrane protein CD63.¹⁷ However as we observed the presence of CD63 in secretory pods previously¹³, it seems unlikely that a connection to the plasma membrane is made during secretory pod formation. The initial rounding and fusion of WPB during secretory pod formation therefore seems to occur prior to exocytosis marking the first steps of multigranular VWF exocytosis.

VWF expelled from secretory pods was found to adopt a globular conformation even in the absence of antibodies, excluding artifacts due to binding of antibodies to VWF. In addition, the globular VWF masses were observed to give rise to VWF strings indicating that VWF string formation is independent from tubular packaging in WPBs. Studies using purified or recombinant VWF multimers also demonstrated that VWF changed from a globular structure into extended chains after applying flow.^{18–20} Our live-cell imaging data indicates that flow has an additional role besides unfolding of VWF. We observed the striking phenomenon that flow induces secreted VWF to move from the side of exocytosis to the edge of the cell where VWF appeared to anchor before forming strings. Data from flow experiments presented by Bouwens et al.²¹ show similar profiles displaying strings emerging from the edge of the cell. Up to date several proteins have been reported to support in VWF anchoring such as P-selectin and the integrin $\alpha\text{v}\beta 3$.^{22,23} However, it is not clear how these proteins contribute to VWF string formation. More players may be involved in VWF anchoring and string formation such as exposed extracellular matrix components like collagen²⁴ or endothelial glycosaminoglycans.^{25,26} Further studies will be required to determine which components guide rolling of globular VWF on the cell surface and which components are involved in VWF anchoring at the edge of the cell.

Based on our data and the current literature we propose a new model of WPB exocytosis, secretory pod formation and VWF remodeling into strings. We postulate that secretory pods are formed by the coalescence of WPBs which pool their content before exocytosis. Clathrin coated vesicles are formed to remove excess membrane while actin stabilizes the secretory pod and guides VWF exocytosis by the contraction of a dynamic ring of actin filaments, myosin II and actomyosin II.²⁷ Upon full collapse of the secretory pod, a large globular mass of VWF is expelled which is moved by fluid flow to the edge of the cell to anchor and unfurl into strings. We show that VWF remodeling into strings is independent of tubular packaging in WPBs and appears to rely on flow which mediates VWF translocation to the edge of the cell, anchoring and unfolding.

Many pathophysiological mechanisms were proposed for various VWD phenotypes, like impaired VWF binding to glycoprotein Ib, loss of high molecular weight multimers, or increased clearance of VWF.⁸ However, impaired VWF string formation due to insufficient anchoring or unfolding of VWF has, until now, not been considered as a cause of VWD.

7.5 Acknowledgments

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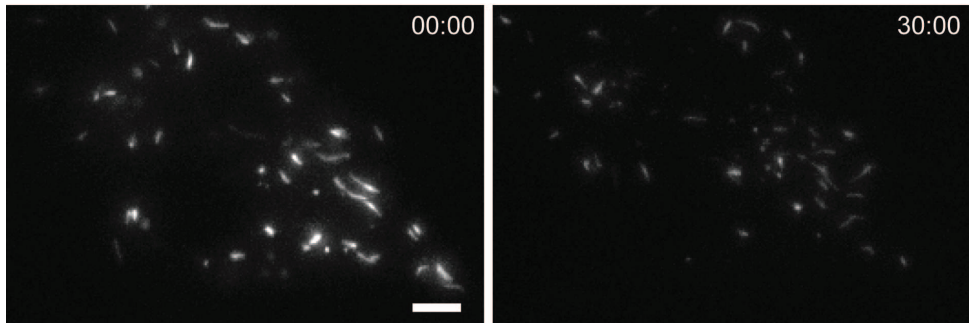
References

- [1] E. R. Weibel and G. E. Palade, *New cytoplasmic components in arterial endothelia*, J Cell Biol **23**, 101 (1964).
- [2] C. J. Lowenstein, C. N. Morrell, and M. Yamakuchi, *Regulation of weibel-palade body exocytosis*, Trends Cardiovasc Med **15**, 302 (2005).
- [3] B. M. Ewenstein, M. J. Warhol, R. I. Handin, and J. S. Pober, *Composition of the von willebrand factor storage organelle (weibel-palade body) isolated from cultured human umbilical vein endothelial cells*, J Cell Biol **104**, 1423 (1987).
- [4] J. H. Reinders, P. G. de Groot, J. J. Sixma, and J. A. van Mourik, *Storage and secretion of von willebrand factor by endothelial cells*, Haemostasis **18**, 246 (1988).
- [5] R. H. Huang, Y. Wang, R. Roth, X. Yu, A. R. Purvis, J. E. Heuser, E. H. Egelman, and J. E. Sadler, *Assembly of weibel-palade body-like tubules from n-terminal*

- domains of von willebrand factor, *Proc Natl Acad Sci U S A* **105**, 482 (2008).
- [6] J. A. Berriman, S. Li, L. J. Hewlett, S. Wasilewski, F. N. Kiskin, T. Carter, M. J. Hannah, and P. B. Rosenthal, *Structural organization of weibel-palade bodies revealed by cryo-em of vitrified endothelial cells*, *Proc Natl Acad Sci U S A* **106**, 17407 (2009).
- [7] J. E. Sadler, *von willebrand factor assembly and secretion*, *J Thromb Haemost* **7 Suppl 1**, 24 (2009).
- [8] K. M. Valentijn, J. E. Sadler, J. A. Valentijn, J. Voorberg, and J. Eikenboom, *Functional architecture of weibel-palade bodies*, *Blood* **117**, 5033 (2011).
- [9] M. G. Rondaij, R. Bierings, A. Kragt, J. A. van Mourik, and J. Voorberg, *Dynamics and plasticity of weibel-palade bodies in endothelial cells*, *Arterioscler Thromb Vasc Biol* **26**, 1002 (2006).
- [10] G. Michaux, K. B. Abbitt, L. M. Collinson, S. L. Haberichter, K. E. Norman, and D. F. Cutler, *The physiological function of von willebrand's factor depends on its tubular storage in endothelial weibel-palade bodies*, *Dev Cell* **10**, 223 (2006).
- [11] J. E. Sadler, P. M. Mannucci, E. Berntorp, N. Bochkov, V. Boulyjenkov, D. Ginsburg, D. Meyer, I. Peake, F. Rodeghiero, and A. Srivastava, *Impact, diagnosis and treatment of von willebrand disease*, *Thromb Haemost* **84**, 160 (2000).
- [12] K. M. Valentijn, J. A. Valentijn, K. A. Jansen, and A. J. Koster, *A new look at weibel-palade body structure in endothelial cells using electron tomography*, *J Struct Biol* **161**, 447 (2008).
- [13] K. M. Valentijn, L. F. van Driel, M. J. Mourik, G. J. Hendriks, T. J. Arends, A. J. Koster, and J. A. Valentijn, *Multi-granular exocytosis of weibel-palade bodies in vascular endothelial cells*, *Blood* **116**, 1807 (2010).
- [14] M. J. Hannah, P. Skehel, M. Erent, L. Knipe, D. Ogden, and T. Carter, *Differential kinetics of cell surface loss of von willebrand factor and its propolypeptide after secretion from weibel-palade bodies in living human endothelial cells*, *J Biol Chem* **280**, 22827 (2005).
- [15] D. N. Mastronarde, *Dual-axis tomography: an approach with alignment methods that preserve resolution*, *J Struct Biol* **120**, 343 (1997).
- [16] J. R. Kremer, D. N. Mastronarde, and J. R. McIntosh, *Computer visualization of three-dimensional image data using imod*, *J Struct Biol* **116**, 71 (1996).
- [17] V. Babich, A. Meli, L. Knipe, J. E. Dempster, P. Skehel, M. J. Hannah, and T. Carter, *Selective release of molecules from weibel-palade bodies during a lingering kiss*, *Blood* **111**, 5282 (2008).
- [18] C. A. Siedlecki, B. J. Lestini, K. K. Kottke-Marchant, S. J. Eppell, D. L. Wilson, and R. E. Marchant, *Shear-dependent changes in the three-dimensional structure of human von willebrand factor*, *Blood* **88**, 2939 (1996).
- [19] A. Barg, R. Ossig, T. Goerge, M. F. Schneider, H. Schillers, H. Oberleithner, and S. W. Schneider, *Soluble plasma-derived von willebrand factor assembles to a haemostatically active filamentous network*, *Thromb Haemost* **97**, 514 (2007).
- [20] S. W. Schneider, S. Nuschele, A. Wixforth, C. Gorzelanny, A. Alexander-

- Katz, R. R. Netz, and M. F. Schneider, *Shear-induced unfolding triggers adhesion of von willebrand factor fibers*, *Proc Natl Acad Sci U S A* **104**, 7899 (2007).
- [21] E. A. Bouwens, M. J. Mourik, M. van den Biggelaar, J. C. Eikenboom, J. Voorberg, K. M. Valentijn, and K. Mertens, *Factor viii alters tubular organization and functional properties of von willebrand factor stored in weibel-palade bodies*, *Blood* **118**, 5947 (2011).
- [22] A. Padilla, J. L. Moake, A. Bernardo, C. Ball, Y. Wang, M. Arya, L. Nolasco, N. Turner, M. C. Berndt, B. Anvari, J. A. Lopez, and J. F. Dong, *P-selectin anchors newly released ultralarge von willebrand factor multimers to the endothelial cell surface*, *Blood* **103**, 2150 (2004).
- [23] J. Huang, R. Roth, J. E. Heuser, and J. E. Sadler, *Integrin $\alpha(v)\beta(3)$ on human endothelial cells binds von willebrand factor strings under fluid shear stress*, *Blood* **113**, 1589 (2009).
- [24] J. H. Rand, N. D. Patel, E. Schwartz, S. L. Zhou, and B. J. Potter, *150-kd von willebrand factor binding protein extracted from human vascular subendothelium is type vi collagen*, *J Clin Invest* **88**, 253 (1991).
- [25] L. J. Fretto, W. E. Fowler, D. R. McCaslin, H. P. Erickson, and P. A. McKee, *Substructure of human von willebrand factor: proteolysis by v8 and characterization of two functional domains*, *J Biol Chem* **261**, 15679 (1986).
- [26] M. Sobel, D. F. Soler, J. C. Kermode, and R. B. Harris, *Localization and characterization of a heparin binding domain peptide of human von willebrand factor*, *J Biol Chem* **267**, 8857 (1992).
- [27] T. D. Nightingale, I. J. White, E. L. Doyle, M. Turmaine, K. J. Harrison-Lavoie, K. F. Webb, L. P. Cramer, and D. F. Cutler, *Actomyosin ii contractility expels von willebrand factor from weibel-palade bodies during exocytosis*, *J Cell Biol* **194**, 613 (2011).

7.A Supplementary material



Suppl. Figure S1. Live-cell imaging snapshots of a control cell expressing propeptide-EGFP. In the control cell no exocytotic events were observed. The GFP signal was strongly reduced as a consequence of photo bleaching. Scale bar, 5 μm .

Video 1. Live-cell imaging of secretory pod formation in propeptide-EGFP transfected cells.

Four WPBs forming a secretory pod. See legend to Figure 7.2 for details. Scale bar, 1 μm .

https://www.dropbox.com/s/o8agas6cq31as1p/ChVII_Video1.mp4?dl=0

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Video 2. Live-cell imaging of secretory pod formation in propeptide-EGFP transfected cells.

Two WPBs forming a secretory pod. See legend to Figure 7.2 for details. Scale bar, 1 μm .

https://www.dropbox.com/s/ama12rws204gmak/ChVII_Video2.mp4?dl=0

Video 3. Segmentation of joined tomograms from five consecutive EM sections.

Tomograms were obtained from the data set presented in Figure 7.3. Colors indicate: Orange, secreted VWF; Pink, secretory pod membrane; Blue, WPB; Yellow, plasma membrane; Green, clathrin coated pits.

https://www.dropbox.com/s/dlo5d8k9g7otxj0/ChVII_Video3.mp4?dl=0

Video 4. Sequence of aligned consecutive TEM sections.

Secretory pods secreting extracellular globules of VWF which give rise to VWF strings. Fluorescence correlating to these structures is projected on top of the EM sections in red. The video is generated from the CLEM data shown in Figure 7.4D.

Scale bar, 500 nm.

https://www.dropbox.com/s/iw8qs0nrt0thp3p/ChVII_Video4.mp4?dl=0

Video 5. VWF exocytosis of propeptide-EGPF transfected cells under flow conditions (2.5 dyne/cm²).

Secreted VWF was labeled by diluted red fluorescent anti-VWF antibodies in the stimulation medium. Arrows indicate emerging strings. Scale bar, 10 μ m.

https://www.dropbox.com/s/lltp89fxswmmkno/ChVII_Video5.mp4?dl=0

