Chapter 6

Analysis of storage and secretion of von Willebrand factor in blood outgrowth endothelial cells derived from von Willebrand disease patients

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Summary

Background: Von Willebrand disease (VWD) patients are often heterozygous for a missense mutation in the von Willebrand factor (*vwf*) gene. Investigating the pathogenic features of VWF mutations in cells directly derived from patients has been challenging.

Methods and results: Here, we have used blood outgrowth endothelial cells (BOECs) isolated from human peripheral blood to analyze the storage and secretion of VWF. BOECs showed full endothelial characteristics and responded to Weibel-Palade body (WPB) secretagogues except desmopressin. We examined BOECs derived from one subject heterozygous for a type 2N mutation (p.Arg854Gln) and from four type 1 VWD patients respectively heterozygous for p.Ser1285Pro, p.Leu1307Pro, p.Tyr1584Cys and p.Cys2693Tyr. Compared to normal BOECs, BOECs heterozygous for p.Ser1285Pro, p.Leu1307Pro or p.Cys2693Tyr showed morphologically abnormal WPB and retention of VWF in the endoplasmic reticulum, whereas BOECs heterozygous for p.Arg854Gln or p.Tyr1584Cys showed normal WPB. Interestingly, a subpopulation of BOECs heterozygous for p.Cys2693Tyr formed normal WPB. The agonist-induced exocytosis of WPB from BOECs and formation of VWF strings on BOECs heterozygous for p.Ser1285Pro, p.Leu1307Pro or p.Cys2693Tyr, but not for p.Arg854Gln or p.Tyr1584Cys, were reduced.

Conclusions: In conclusion, VWD phenotype can be recapitulated in BOECs and thus BOECs provide a feasible *bona fide* cell model to study the pathogenic effects of VWF mutations.

Introduction

Von Willebrand disease (VWD) is the most common inherited human bleeding disorder. It is mainly caused by mutations in the von Willebrand factor (*vwf*) gene that encodes von Willebrand factor (VWF), a large multimeric, hemostatic protein [1]. VWF supports primary hemostasis by recruiting platelets to the subendothelial matrix or endothelial cell surface upon vascular perturbation or injury. Furthermore, VWF binds coagulation factor VIII (FVIII) to protect it from premature proteolysis in the circulation [2,3]. In endothelial cells VWF is stored in equimolar amounts with VWF propeptide (VWFpp) in Weibel-Palade bodies (WPB) for basal and regulated secretion [4,5]. Upon exocytosis of WPB, VWF is released and forms ultra-long, hyper-adhesive strings that stay attached to endothelial cells while VWFpp rapidly diffuses into the blood. VWF strings bind platelets from flowing blood to initiate clot formation and then the strings are rapidly cleaved into smaller VWF multimers by ADAMTS-13 to avoid vascular occlusion [6]. Defects in the formation or function of VWF strings may lead to a bleeding tendency as seen in VWD.

VWD has been classified into three categories: type 1 and type 3 with quantitative deficiency of VWF and type 2 with functional defects [1]. Type 1 VWD is the most common type (up to 75% of all VWD cases) with reduced VWF levels caused by multiple factors including inefficient synthesis, storage or secretion, endoplasmic reticulum (ER) related intracellular degradation or faster clearance from the circulation [7,8]. The mutations identified in type 1 VWD are heterogeneous and located throughout the whole gene [9]. Although several VWF mutations identified in type 1 VWD have been extensively studied in heterologous cell systems [7], interpreting the phenotypic defects of such mutations is not always easy. One of the limitations is the fact that in most cell model-systems such as COS cells, VWF is not intracellularly stored [10]. Only few cell lines such as HEK293 cells form pseudo-WPB and are useful for analyzing the structure-function relation of VWF. Studies in these cell lines have provided insights into the effects of VWF mutations on the storage and secretion of VWF [11-17]. On the other hand, the use of non-endothelial cell systems has obvious limitations. The possible gross overexpression of recombinant VWF upon transfection may influence interpretation of the data and the exocytotic machinery is probably different from that of endothelial cells. Mimicking the heterozygous state of VWF mutations by co-transfection of wild type and mutant VWF is potentially also problematic.

Human umbilical vein endothelial cells (HUVECs) have been derived from VWD patients and been characterized many years ago [18-22]. However, HUVECs can

not be widely used for such studies because the access to HUVECs with specific VWF mutations is extremely limited.

Blood outgrowth endothelial cells (BOECs) may provide an alternative for HUVECs. BOECs can be derived from circulating endothelial progenitor cells upon culture of human peripheral- or cord blood mononuclear cells [23,24]. In culture, BOECs have all the endothelial characteristics including expression of endothelial cell markers and capability of forming capillary-like structures in Matrigel [24]. It has been suggested that BOECs provide a useful system to study release of WPB and formation of VWF strings [25]. In addition, morphology of WPB in the BOECs derived from one VWD patient has been characterized in a recent study [17]. The aim of this study was to further explore the feasibility of using BOECs as an endothelial cell model to study the pathogenic nature of VWF mutations at the molecular cellular level. To this end, we isolated BOECs from five healthy donors and five subjects carrying VWF mutations and characterized these cells. Furthermore, we tested the response of BOECs to different WPB secretagogues and analyzed VWF storage in and regulated secretion from VWD BOECs.

Patients, materials and methods

Patients

VWD patients heterozygous for VWF mutations p.Ser1285Pro, p.Leu1307Pro, p.Tyr1584Cys and p.Cys2693Tyr, respectively, were enrolled in this study (Table 1). All four patients were initially diagnosed with type 1 VWD. One asymptomatic participant was heterozygous for the type 2N VWF mutation p.Arg854Gln. Plasma was prepared and tested using standard procedures for FVIII and VWF evaluation, including FVIII:C, VWF:Ag, VWF:RCo, VWF:CB and VWF multimer profile. The study protocol was approved by the Leiden University Medical Center ethics review board. Informed consent was obtained from all subjects in accordance with the Declaration of Helsinki.

BOECs isolation and culture

BOECs were isolated and cultured as previously described [23] with modifications. 80 mL of venous blood from each subject was collected in S-Monovette tubes (S-Monovette® 10 ml, Coagulation 9 NC, containing 0.1 volume 0.106 M trisodium citrate, Sarstedt, Nümbrecht, Germany). The citrated blood was diluted 1:1 with Ca^{2+}- and Mg^{2+}-free HBSS (Invitrogen, Carlsbad, CA, USA). Buffy coat mononuclear cells were isolated by gradient centrifugation over Ficoll-Paque PLUS

(GE Healthcare, Diegem, Belgium) at 800 *g* for 30 minutes and washed twice at 700 *g* for 10 minutes. Cell pellets were resuspended in EGM-2 medium (Lonza, Breda, The Netherlands) supplemented with 20% fetal bovine serum, a cocktail of growth factors, 100 U/mL penicillin, 100 µg/mL streptomycin and 250 ng/mL amphotericin (Invitrogen) and seeded onto 6-well tissue culture plates pre-coated with rat tail collagen type I (BD Biosciences, Bedford, MA, USA). Culture medium was refreshed daily until the first colony appeared, then medium was refreshed every two days. Cells were used at passages 3 to 8 in all experiments unless stated otherwise.

FACS analysis

BOECs were washed once with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.01% sodium azide and incubated with the primary antibody or isotype control for 30 min at room temperature in the dark. We used primary mouse monoclonal antibodies (all purchased from BD Biosciences, unless stated otherwise) against human CD14 conjugated to fluorescein isothiocyanate (FITC), human CD45 conjugated to Peridinin-chlorophyll proteins (PerCP), human CD31 conjugated to phycoerythrin (PE), human CD34 and human CD133 conjugated to allophycocyanin (APC). Monoclonal antibody against endothelial protein C receptor (EPCR, unlabeled) was kindly provided by Prof. Dr C. T. Esmon (Howard Hughes Medical Institute, USA). A secondary antibody conjugated to Alexa Fluor 647 (Invitrogen) was used to label EPCR. In separate tubes, cells were incubated with isotype controls. Samples were fixed in 1% paraformaldehyde and analyzed by flow cytometry (FACS LSRII, BD Biosciences) within 24 hours. Data were analyzed using FACSDiVa software (BD Biosciences).

Matrigel assay and microscopic imaging

Matrigel assays were performed according to the manufacturer's instructions. Matrigel (BD Biosciences) was thawed overnight on ice. Ninety-six-well tissue culture plates were coated with 30 µL/well Matrigel for 30 minutes at 37°C. BOECs were suspended in complete EGM-2 medium and seeded for 4-6 hours at a cell density of 5,000 to 20,000 cells per well. Images were taken by Leica DM IL LED inverted microscopy equipped with the Leica DFC295 digital camera (Leica Microsystems).

Confocal immunofluorescence microscopy

BOECs were grown on coverslips coated with collagen type I. Confluent cells were fixed, permeabilized and stained essentially as previously described [11]. To visualize VWF strings, confluent cells were stimulated as indicated prior to fixation. Monoclonal antibody CLB-RAg35 [26] and polyclonal antibody rabbit anti-human VWF (DAKO, Glostrup, Denmark) were used to visualize VWF. Monoclonal antibody CD62P (Clone AC1.2, BD Biosciences), polyclonal antibody rabbit anti-human β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and polyclonal antibody rabbit anti-human Protein Disulfide Isomerase (PDI) antibody A66 (obtained from Prof. I. Braakman, Department of Chemistry, Utrecht University, Utrecht, The Netherlands) were used to visualize P-selectin, β-catenin and the ER marker PDI, respectively. Alexa 488- and Alexa 594-conjugated secondary antibodies were purchased from Invitrogen. Samples were analyzed by Leica SL confocal laser scanning microscopy with a 63X/1.40 NA oil objective (Leica Microsystems).

Stimulation of Weibel-Palade body exocytosis

BOECs were grown on collagen type I coated coverslips or in collagen type I coated 12-well tissue culture plates. Confluent cells were stimulated for one hour with 160 nM phorbol-12-myristate-13-acetate (PMA), 100 µM histamine, 0.05-1 µM DDAVP (desmopressin acetate 3-water) or 10 µM epinephrine plus 100 µM isobutylmethylxanthine (IBMX) in serum-free medium (OPTIMEM1 medium [Invitrogen], 10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 0.2% bovine serum albumin, pH 7.4). DDAVP was purchased from Ferring Pharmaceuticals (Hoofddorp, the Netherlands). PMA, histamine, epinephrine and IBMX were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Quantification of VWF and VWFpp

VWF antigen [11] and VWFpp [27] were measured essentially as described before with minor modifications. Polyclonal antibody rabbit anti-human VWF and polyclonal antibody goat anti-rabbit IgG coupled to peroxidase (DAKO) were used to measure VWF antigen. Monoclonal antibody mouse anti-human VWFpp CLB-Pro 35 and CLB-Pro 14.3 coupled to peroxidase (Sanquin, Amsterdam, The Netherlands) were used to measure VWFpp. Normal pooled human plasma was used as the reference.

To quantify basal secretion of VWF, confluent cells in 12-wells were incubated at 37°C in EGM-2 medium (containing 2% fetal bovine serum and supplemented with a cocktail of growth factors and antibiotics as described above). On the basis of previous work by Giblin et al [5], the conditioned media were collected after incubation for 5 hours. VWF:Ag secreted into the conditioned media in 5 hours was defined as basal secretion of VWF. Cells were lysed overnight at 4°C with serum-free medium containing 0.1% Triton-X100 and the protease inhibitor cocktail Complete™ with EDTA (Roche Diagnostics, Mannheim, Germany). Cell lysates were vortexed for 10 seconds before centrifugation. The supernatants of conditioned media and cell lysates were collected after centrifugation at 14,000 rpm for 5 minutes. VWF:Ag in the medium and lysate was measured by ELISA.
To quantify the release of VWF or VWFpp during exocytosis of WPB, the conditioned media and lysates were collected after stimulation as described above. All the supernatants were supplemented with phenylmethylsulfonyl fluoride (PMSF) (Roche) at a final concentration of 100 μM, and then analyzed immediately or snap-frozen. The percentage of secreted VWF:Ag or VWFpp during stimulation was defined as regulated secretion and was expressed as a fraction of total amount of antigen (% of total = absolute antigen in the medium/(absolute antigen in the medium + absolute antigen in the cell lysate) × 100).

Multimer analysis
Frozen plasma was thawed at 37 °C and centrifuged for 5 minutes at 14,000 rpm. The supernatants were collected for VWF multimer analysis. BOECs were cultured until confluent and then incubated with EGM-2 medium (containing 2% serum) for 24 hours. The supernatants of conditioned media and lysates of BOECs were collected as described above. VWF multimers were separated by non-reducing 1.6% agarose gel electrophoresis with sodium dodecyl sulfate (SDS) and visualized by Western blotting as described [28].

Results

Phenotypic and genotypic characterization of VWD patients
The four VWD patients are heterozygous for VWF mutations p.Ser1285Pro, p.Leu1307Pro, p.Tyr1584Cys and p.Cys2693Tyr, respectively. All the patients are female and have blood group O (Table 1). They were historically diagnosed as type 1 VWD based on low plasma levels of VWF and nearly normal VWF multimer patterns [29]. Detailed analysis of VWF multimers identified marginal decrease of

the largest VWF multimers in the patients heterozygous for VWF mutations p.Ser1285Pro and p.Leu1307Pro [30]. Based on the 2006 classification criteria p.Ser1285Pro fits the diagnosis of type 1, whereas p.Leu1307Pro might be reclassified as type 2A [1,30]. The asymptomatic subject heterozygous for the type 2N mutation p.Arg854Gln showed a reduced binding ability to FVIII but normal level of VWF (Table 1).

Characteristics of BOECs

BOECs were derived from peripheral blood mononuclear cells. After 2~4 weeks culture, colonies with cobblestone-like cells appeared (Figure 1A). These cells were late outgrowth endothelial cells, referred to as BOECs in this manuscript. Immunofluorescence microscopy showed that VWF was stored by BOECs in elongated organelles (Figure 1B) that displayed internal striations under electron microscopy (data not shown) [25]. Furthermore, these organelles also stored the WPB-specific membrane protein P-selectin (Figure 1B). These data indicate that VWF was stored in authentic WPB.

Functional analysis showed that BOECs were able to form capillary-like structures in Matrigel (Figure 1C).

FACS analysis (Figure 1D-M) showed that BOECs were positive for the endothelial cell marker CD31 (PECAM-1) and the endothelial protein C receptor (EPCR), and negative for leukocyte markers CD14 and CD45. In addition, part of the BOECs were positive for the stem cell marker CD34 (~70%) but negative for CD133. This indicates that BOECs have differentiated to mature endothelial cells. The histamine receptor H1 mediates histamine-induced secretion of VWF from endothelial cells [31]. By real time PCR we found that in BOECs H1 receptor is highly expressed while H2 receptor is expressed at a very low level. Expression of H3 and H4 receptors in BOECs was not detectable (data not shown).

The yield of BOEC colonies was donor-dependent. So far we failed to isolate BOECs from one healthy donor and one VWD patient heterozygous for p.Cys1149Arg. Several attempts to establish BOECs from these two subjects were unsuccessful, while BOECs were obtained from two healthy donors (donor 2 and donor 3 in the current study) in each of four different attempts.

Table 1. Characteristics of investigated subjects

Subject	Gender	Blood group	Nucleotide change	Amino acid change	VWF:Ag, IU/dL	VWF:RCo, IU/dL	VWF:RCo/ VWF:Ag	FVIII:C, IU/dL	VWF:CB, IU/dL	Multimer pattern	VWD type*
P6F1III1†	Female	O/O	c.2561G>A	p.Arg854Gln	73	114	1.56	59	Not tested	Normal	2N
P6F3II1§	Female	O/O	c.3853T>C	p.Ser1285Pro	16	3	0.19	27	8	Abnormal‡	1
P6F13II1§	Female	O/O	c.3920T>C	p.Leu1307Pro	16	7	0.44	33	6	Abnormal	2A
P6F12II1§	Female	O/O	c.4751A>G	p.Tyr1584Cys	48	36	0.75	64	52	Normal	1
P6F16II1§	Female	O/O	c.8078G>A	p.Cys2693Tyr	46	64	1.39	69	42	Normal	1

*VWD type according to the 2006 classification [1].

†Asymptomatic subject.

‡The multimer abnormality is minimal and fits classification of VWD type 1 [30].

§Characteristics of these four subjects have been reported previously [29].

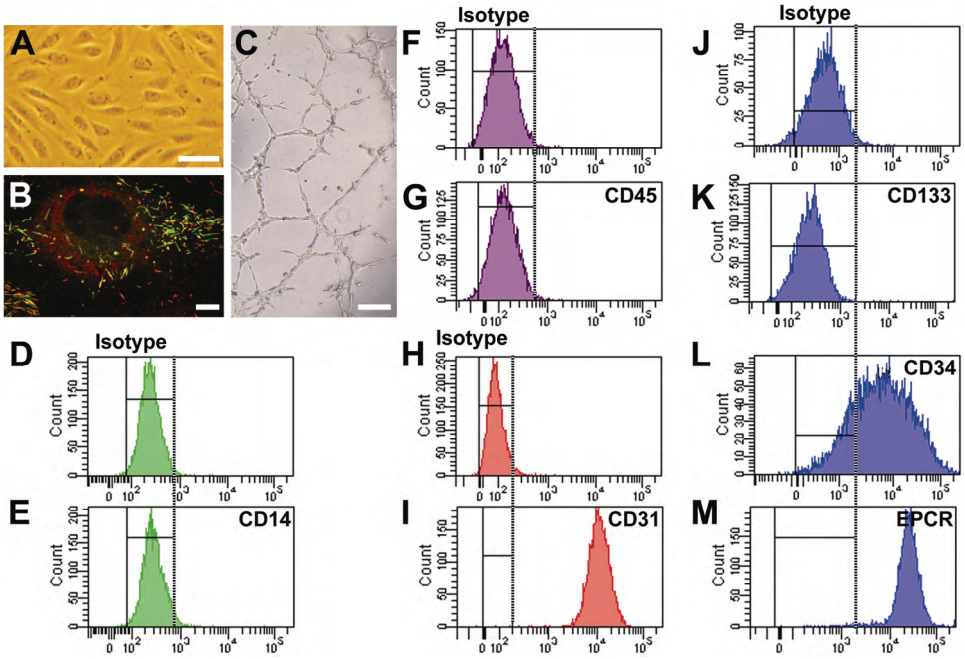

Figure 1. Phenotypic and functional analysis of BOECs. (A) Cobblestone-like morphology of BOECs under bright field microscopy. Scale bar represents 100µm. (B) BOECs store VWF (shown in red) and P-selectin (shown in green) in WPB. Yellow indicates the co-storage of VWF and P-selectin in WPB (elongated organelles). Image was taken by Leica SL confocal laser scanning microscopy with a 63X/1.40 NA oil objective. Scale bar represents 10 µm. (C) BOECs formed capillary-like structures in Matrigel. Scale bar represents 200µm. (D-M) FACS analysis of BOECs derived from one healthy donor. The representative data demonstrated that BOECs were negative for leukocyte cell makers CD14 (D = isotype, E = CD14) and CD45 (F = isotype, G = CD45) but positive for endothelial cell markers CD31 (PECAM-1, H = isotype, I = CD31) and EPCR (J = isotype, M = EPCR). In addition, BOECs completely lost expression of the stem cell maker CD133 (J = isotype, K = CD133) though the majority of cells still express the progenitor cell marker CD34 (J = isotype, L = CD34). Shown are representative data from at least 4 independent experiments using BOECs derived from one healthy donor. Cells derived from other healthy donors and the patients showed similar results.

Storage of VWF in BOECs derived from VWD patients

VWF drives the biogenesis of WPB. Mutations in VWF may disturb the formation and function of WPB. VWF was stored normally in elongated WPB in BOECs derived from healthy donors and from subjects with VWF mutations p.Arg854Gln and p.Tyr1584Cys (Figure 2A-C). Mainly short/round WPB were observed in BOECs derived from patients with VWF mutations p.Ser1285Pro, p.Leu1307Pro

and p.Cys2693Tyr (Figure 2D-F). Abundant reticular VWF staining was observed in BOECs heterozygous for the latter three VWF mutations. Further analysis showed that the diffuse VWF staining co-localized with the ER marker PDI indicating retention of VWF in the ER (Figure 2G). The BOECs derived from the patient heterozygous for p.Cys2693Tyr contained a small cell population (less than 10% of the total cells) in which VWF was stored normally in elongated WPB but without significant retention in the ER (Figure 2F'). These two distinct cell populations co-existed regardless of culture passage number, clone they were derived from, or the confluency of the cells.

Production and basal secretion of VWF from BOECs

Production and basal secretion of VWF were examined for BOECs (Table 2). Compared with that of healthy donors, the total VWF production was much lower for BOECs with VWF mutations p.Ser1285Pro and p.Leu1307Pro, while it was similar for BOECs with p.Arg854Gln, p.Tyr1584Cys or p.Cys2693Tyr. Basal secretion of VWF was in the normal range for BOECs with VWF mutation p.Tyr1584Cys and was slightly lower for BOECs with VWF mutations p.Arg854Gln and p.Cys2693Tyr. For BOECs with VWF mutations p.Ser1285Pro or p.Leu1307Pro, VWF basal secretion was drastically decreased (3-5 mU versus 20-66 mU).

Table 2. Basal secretion of VWF from BOECs

Subject	Amino acid change	Total VWF production (mU)	VWF in medium (mU)
Healthy donor 1	-	175.5 ± 0.8	61.7 ± 0.7
Healthy donor 2	-	98.2 ± 10.2	66.2 ± 5.9
Healthy donor 3	-	41.5 ± 1.0	20.5 ± 1.3
Healthy donor 4	-	128.1 ± 6.9	42.8 ±1.6
Healthy donor 5	-	181.8 ± 4.6	48.8 ± 3.5
P6F1III1	p.Arg854Gln	82.2 ± 1.2	18.7 ± 0.4
P6F3II1	p.Ser1285Pro	16.4 ± 1.1	5.4 ± 0.2
P6F13II1	p.Leu1307Pro	5.7 ± 0.5	3.0 ± 0.3
P6F12II1	p.Tyr1584Cys	155.5 ± 35.1	45.4 ± 1.2
P6F16II1	p.Cys2693Tyr	121.5 ± 0.0	16.0± 0.1

VWF:Ag was determined for all BOECs at passage 6. Cells were grown until confluent in 12-well plates (approximately $1x10^5$ cells per well) and incubated for 5 hours with EGM-2 medium (containing 2% serum). Total VWF production = VWF:Ag in conditioned medium (basal secretion) + VWF:Ag in the lysate. Each value represents the mean ± SEM of duplicate or triplicate measurements.

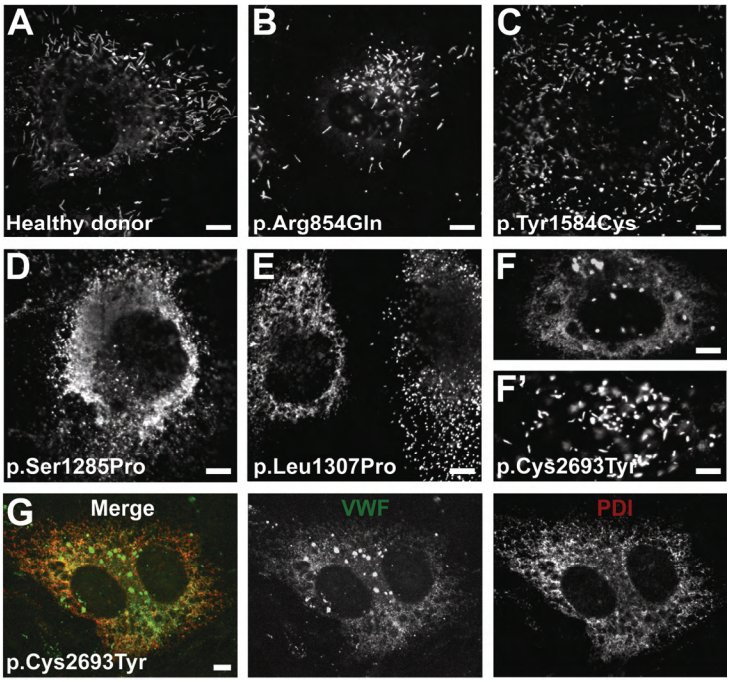

Figure 2. Storage of VWF in BOECs. BOECs derived from a healthy donor or patients with mutations as indicated were grown on collagen type 1 pre-coated cover slips until confluent. (A-F') Cells were fixed and stained for VWF. The punctate staining indicates storage of VWF in WPB while the diffuse staining in panels D-F indicates VWF retained within the ER. (G) A representative image shows that the diffuse VWF staining observed in BOECs derived from the patients heterozygous for p.Ser1285Pro, p.Leu1307Pro and p.Cys2693Tyr was in the ER. BOECs were fixed and stained for VWF (middle panel, green) and the ER marker PDI (right panel, red). In the left panel (merge of green and red channels) the WPB show up in green (VWF staining only), and the ER containing VWF shows up in yellow as a result of double staining for VWF and PDI. Note that the majority of WPB observed in BOECs derived from the patients heterozygous for VWF p.Ser1285Pro, p.Leu1307Pro and p.Cys2693Tyr were short or round instead of elongated as seen in panel A. A fraction of cells derived from the patient heterozygous for p.Cys2693Tyr showed completely normal morphology of WPB (F'). Scale bars represent 5 µm. All images were taken by Leica SL confocal laser scanning microscopy with a 63X/1.40 NA oil objective.

Distribution of VWF multimers from BOECs

To correlate the phenotype of the BOECs to that of the patients, we also compared the VWF multimers from both BOECs and patients' plasma (Figure 3). In agreement with our previous reports [29,30], plasma VWF from the patients with p.S1285Pro and p.Leu1307Pro showed a slightly abnormal multimer pattern with a marginal loss of the largest multimers, while plasma VWF from the patients with p.Arg854Gln, p.Tyr1584Cys and p.Cys2693Tyr [29] showed a normal multimer pattern (Figure 3A). The multimer patterns of plasma VWF from all healthy donors were normal. Indeed VWF secreted from BOECs derived from healthy individuals and mutation carriers with p.Arg854Gln, p.Tyr1584Cys and p.Cys2693Tyr showed a normal multimer pattern (Figure 3B). VWF secreted from BOECs derived from the patients with p.S1285Pro and p.Leu1307Pro mutations showed partial loss of largest multimers as was also seen in plasma. In the medium of the BOECs higher molecular weight multimers were present compared to normal pooled plasma (lane NPP). This is explained by the insufficient, if any, proteolysis by ADAMTS-13 and absence of clearance in the experimental conditions of the BOECs culture. The VWF multimer patterns in cell lysates were similar to the corresponding multimer patterns in medium (Figure 3B-C).

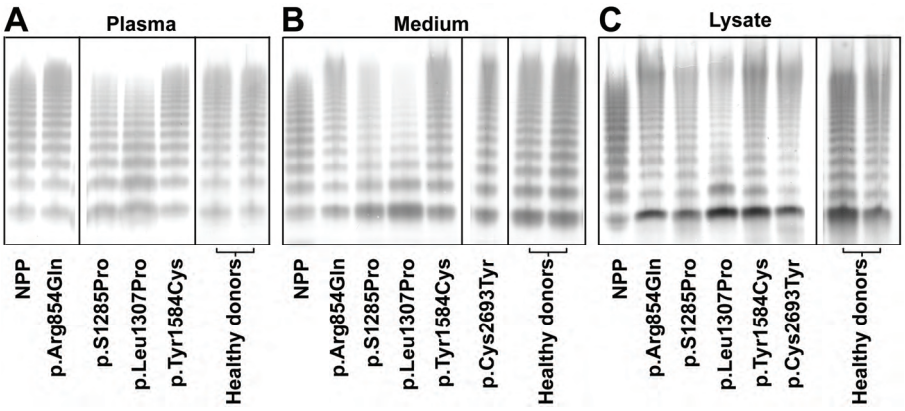

Figure 3. Multimer analysis of VWF. Multimers are shown for VWF in the plasma from indicated subjects (A), in the culture medium (B) or in the cell lysates (C) of BOECs. The lanes separated by the black lines are representative results from the same gels or from different experiments. Normal pooled plasma (NPP) was used as reference. The plasma of the patient heterozygous for p.Cys2693Tyr was unfortunately no longer available, but the multimer pattern for this patient has been reported before as normal.[29] VWF multimer pattern of all healthy donors was normal, only two representative patterns are shown. The multimer patterns of VWF were analyzed by SDS-agarose gel electrophoresis and Western blot under non-reducing conditions.

Regulated secretion of VWF and VWFpp

VWF and VWFpp are stored together in WPB for both basal and regulated secretion. To quantify exocytosis of WPB, we determined the secretion of both VWF and VWFpp. Firstly we examined the variations of VWF and VWFpp secretion and production from BOECs between culture passages and clones. As shown in Figure 4 for BOECs from a healthy donor and in supplemental Figure S1 for BOECs from the patient heterozygous for p.Tyr1584Cys, the regulated secretion of VWFpp, the relative secretion induced by PMA, i.e. the fraction of total amount of VWFpp, was rather stable within the first 10 culture passages. The regulated secretion of VWF from BOECs heterozygous for p.Tyr1584Cys varied and did not reflect the extent of exocytosis very well (Supplemental Figure S1A). The absolute production of VWF and VWFpp was only constant within culture passages 5 to 7 for both BOECs. While the absolute production of VWFpp could vary to a large extent, the regulated secretion of VWFpp was rather constant between different clones of BOECs (Supplemental Figure S2). Furthermore, that the relative secretion of VWFpp during stimulation was at least two folds higher than that of VWF:Ag made it easier to detect exocytosis of WPB [32]. Taken together, these results suggest that it is more reliable to determine VWF production at early culture passages of BOECs (as done in Table 2) and that measuring VWFpp is better suited than determining VWF:Ag as a measure of WPB exocytosis. Therefore, we determined the percentage of secreted VWFpp to quantify exocytosis of BOECs.

We examined the exocytosis of WPB from BOECs by induction with different agonists. For BOECs derived from healthy donors (n=5), 40-60% VWFpp was released to the medium upon stimulation with 160 nM PMA or 100 µM histamine for 60 minutes, while 30-50% VWFpp was released upon stimulation with 10 µM epinephrine for 60 minutes (Figure 5A). BOECs derived from the patient with p.Arg854Gln showed a completely normal response to all the three agonists, while the responses of BOECs derived from the patients with p.Ser1285Pro, p.Leu1307Pro or p.Cys2693Tyr were reduced (Figure 5B-D). The response to PMA of BOECs derived from the patient with p.Tyr1584Cys was slightly, but significantly, reduced while the response to histamine and epinephrine was in the normal range. The relatively milder impairment in WPB exocytosis of BOECs with p.Cys2693Tyr compared with that of BOECs with p.Leu1307Pro is consistent with the responses of the two patients to DDAVP infusion (Table 3). The relative increase after DDAVP infusion is larger for the patient with p.Leu1307Pro (3 times increase for p.Leu1307Pro versus 2 times increase for p.Cys2693Tyr). This is probably

because of the low baseline level of plasma VWF (under steady state) due to the faster clearance of VWF variant p.Leu1307Pro in the patient [33].

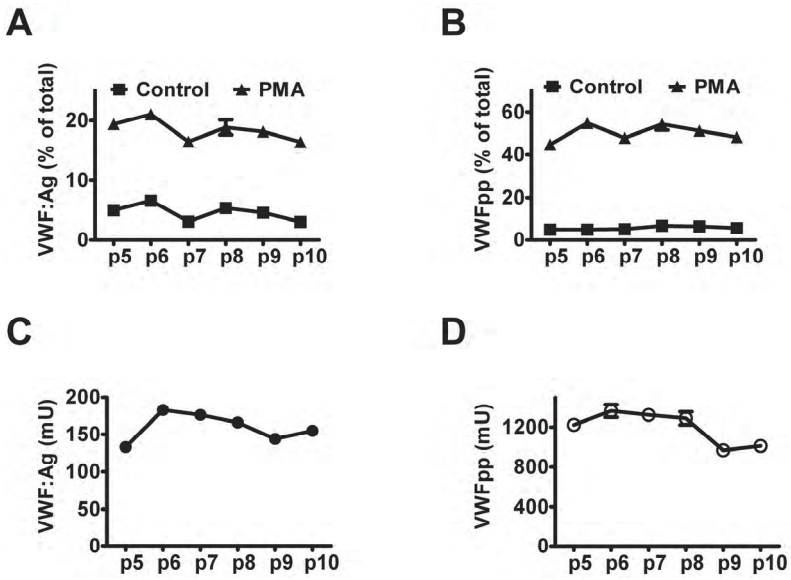

Figure 4. Regulated secretion of VWF and VWFpp from BOECs. BOECs derived from a healthy donor were cultured in 12-well plates until confluent. After rinsing with HBSS, cells were incubated for 60 minutes without (Control) or with 160 nM PMA (PMA) at 37 °C. The regulated secretion of VWF (A) and VWFpp (B) was determined for BOECs at given passages. In panels C and D, the Y-axis indicates the total production (medium plus lysate) of VWF or VWFpp by approximate $1x10^5$ cells. Mean ± SEM indicate variation between triplicate measurements.

Table 3. DDAVP response of VWF:Ag levels

Subject	Amino acid change	DDAVP administration	VWF:Ag, IU/dL
P6F13II1	p.Leu1307Pro	Before	14
		1 hour	48
P6F16II1	p.Cys2693Tyr	Before	48
		1 hour	90
		2 hours	101
		4 hours	85

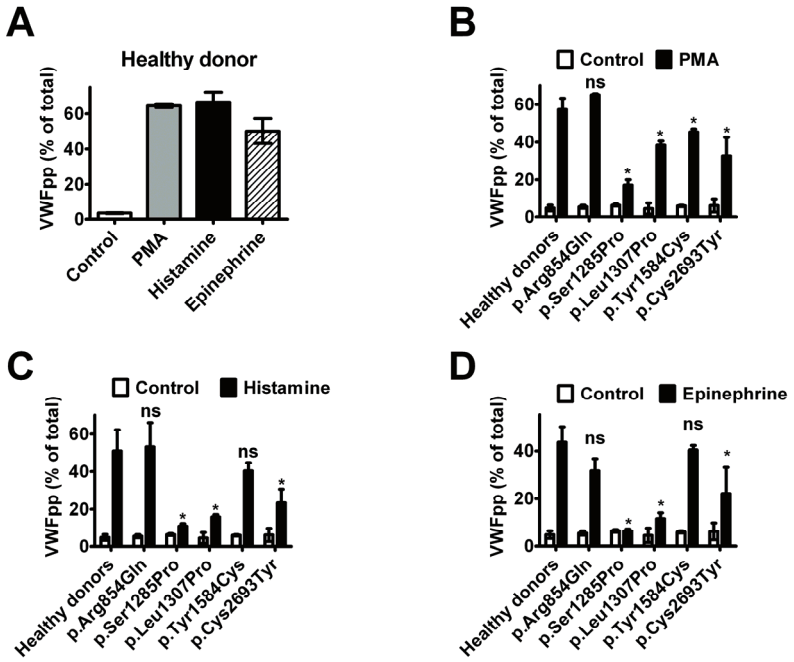

Figure 5. Exocytosis of WPB of BOECs. BOECs were cultured in 12-well plates until confluent and then rinsed and incubated for 60 minutes at 37 °C with release medium in the absence (Control) or presence of 160 nM PMA (PMA), 100 µM histamine (Histamine) or 10 µM epinephrine plus 100 µM IBMX (Epinephrine). Each bar represents the percentage of secreted VWFpp as a fraction of total VWFpp (medium plus lysate) by three different passages of BOECs (between passages 4 to 8). (A) Regulated secretion of VWFpp from BOECs of one representative healthy donor. Mean ± S.D. indicates variation of VWFpp secretion between three passages of BOECs. (B-D) Regulated secretion of VWFpp induced by PMA, histamine or epinephrine was compared between BOECs derived from healthy donors (mean of five donors) and five subjects heterozygous for a given VWF mutation. Mean ± S.D. indicates variation of VWFpp secretion between three passages of one clone of BOECs (clone A for healthy donor 2 and clone A for donor 5 as presented in Figure S2). The bars for healthy donors in panels B-D were generated by pooling data from five healthy donors. In this case, the error bars indicate S.D. between the five healthy donors. *Student's *t*-test $p<0.05$. ns: the difference is statistically not significant.

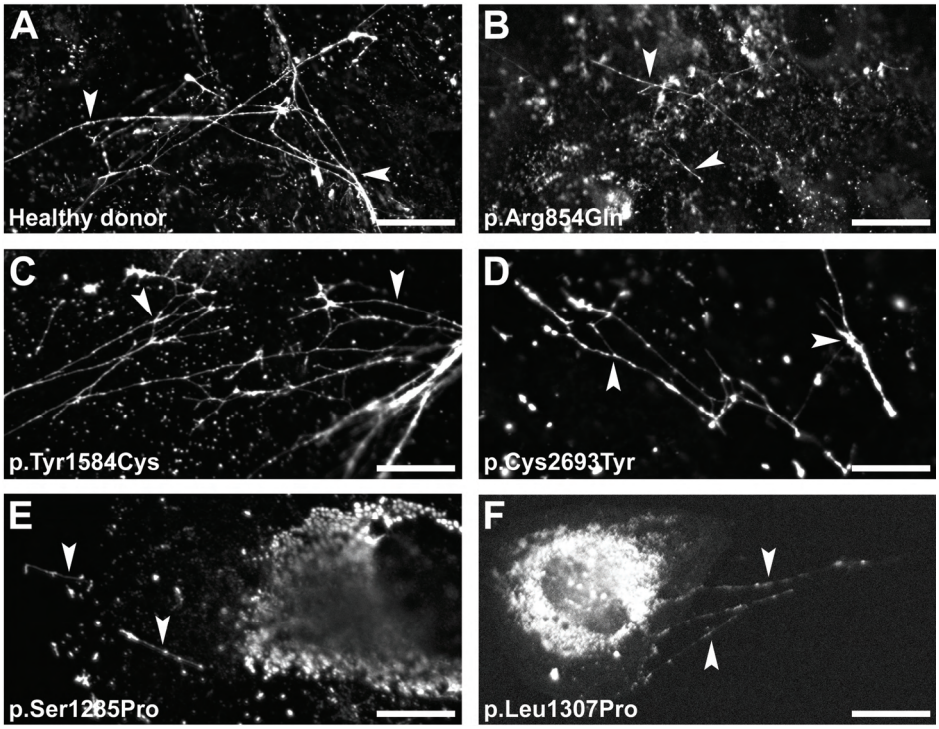

Figure 6. VWF string formation upon exocytosis of WPB. BOECs derived from a healthy donor (A) or from the subjects heterozygous for a VWF mutation (B-F) were stimulated for 60 minutes with 100 µM histamine at 37 °C. Cells were fixed and stained for VWF to visualize the VWF string-like structures (indicated by arrowheads). Scale bars represent 20 µm. All images were taken by Leica SL confocal laser scanning microscopy with a 63X/1.40 NA oil objective.

VWF string formation on the cell surface of BOECs

VWF strings readily appeared on the cell surface upon stimulation with histamine (Figure 6A). BOECs derived from the patients with p.Arg854Gln, p.Tyr1584Cys or p.Cys2693Tyr released VWF strings which were similar to that released from healthy BOECs (Figure 6B-D). BOECs with p.Cys2693Tyr showed less VWF strings. Only a few, short, VWF strings were observed for BOECs derived from the patients with mutation p.Ser1285Pro or p.Leu1307Pro (Figure 6E-F). Compared to BOECs derived from other subjects, the BOECs derived from these two patients showed considerable amounts of VWF remaining within the cells after stimulation. This indicates that large amounts of VWF were not stored in WPB, as shown in Figure 2. Epinephrine also induced formation of VWF strings (Supplemental Figure

S3). Similar to previous observations in HUVECs [26], epinephrine induced clustering of remaining WPB to the perinuclear region of the cell. Stimulation with PMA (data not shown) of BOECs derived from the corresponding subjects showed similar results to that presented in Figure 6.

Since DDAVP is assumed to induce exocytosis of WPB via the vasopressin V2 receptors in endothelial cells [34,35], we tested whether DDAVP response can be used in BOECs. We observed that BOECs did not respond to DDAVP treatment (Figure 7). These results indicate that BOECs probably do not express V2 receptors.

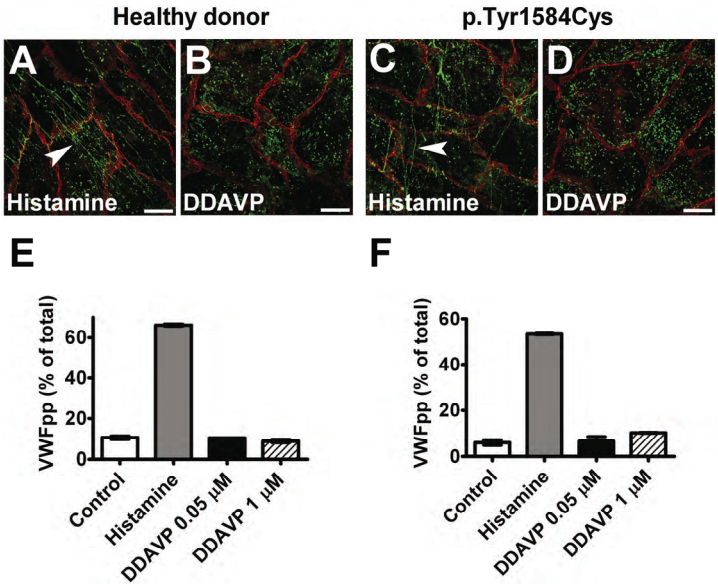

Figure 7. Response of BOECs to DDAVP. BOECs derived from a healthy donor (A-B) or the patient heterozygous for p.Tyr1584Cys (C-D) showed VWF strings (indicated by arrowheads) after incubation with 100 µM histamine for 1 hour (A and C), but no strings after stimulation with 1µM DDAVP for 2 hours (B and D). Cells were fixed and stained for VWF (green) and β-catenin (red). Scale bars represent 20 µm. All images were taken by Leica SL confocal laser scanning microscopy with a 63X/1.40 NA oil objective. (E-F) Cells derived from a healthy donor (donor 1) (E) and from the patient heterozygous for p.Tyr1584Cys (F) were cultured in 12-well plates at passage 6. Confluent cell layers were incubated for 60 minutes at 37 °C in the absence (Control) or presence of 100 µM histamine (Histamine) or DDAVP at indicated final concentrations. There was no response to DDAVP. Mean ± SEM indicate variation between triplicate measurements.

Discussion

In this study, we examined whether human peripheral blood-derived BOECs are useful for the investigation of VWF mutations in their natural environment, in particular with respect to intracellular storage and regulated secretion of VWF. We showed that isolation and culturing of BOECs from multiple VWD patients was feasible. Using such BOECs, we demonstrated that VWF mutations p.Ser1285Pro, p.Leu1307Pro and p.Cys2693Tyr led to intracellular retention of VWF and consequently to impairment in the formation and exocytosis of WPB and to a decrease in the formation of VWF strings upon stimulation of the cells. VWF mutation p.Tyr1584Cys only showed marginal defects and the type 2N mutation p.Arg854Gln did not show defects in these processes. The defective biogenesis and exocytosis of WPB shown in BOECs most likely contribute to the quantitative deficiency of VWF in these patients.

Ectopic expression of VWF in some heterologous cell systems has advanced our understanding of the pathogenic nature of VWF mutations including defects in assembly of VWF multimers, increase retention and/or degradation of VWF in the ER and impairment in VWF storage (formation of pseudo-WPB) [11,12,28,36-38]. However, confirming the pathogenic nature of type 1 VWD mutations by expression studies is inherently difficult. Firstly, it is difficult to reliably measure quantitative defects in *in vitro* transient expression systems. Secondly, most type 1 VWD patients are heterozygous for VWF mutations and it is difficult to exactly mimic the heterozygous state in co-transfection experiments. Thirdly, most expression studies use heterologous cell systems instead of endothelial cells [29,39-41].

The advantages of using BOECs as an *in vitro* model are obvious. The expression of endothelial cell markers, the presence of the endothelial-specific WPB and the capability to form capillary-like structures in Matrigel clearly demonstrated that BOECs were authentic endothelial cells. Transfection of VWF in non-endothelial cell lines is a useful, valid and accessible approach to study VWF mutants, however, with the use of BOECs some of the drawbacks inherent to heterologous expression can be eliminated. For example, ectopic expression may lead to gross overexpression of VWF and different posttranslational modifications compared to endothelial cells. In addition, in co-transfection experiments the proportion of expressed wild-type and mutant VWF may not reflect the expression of mutant VWF in the patients. Reduced expression or production of VWF mutant protein compared to the wild type allele is not uncommon and several studies have shown that alternative splicing induced instability and premature degradation of mRNA

contributes to low production of VWF from mutated alleles [17,42-44]. This mechanism may explain the co-existence of heterogeneous cell populations derived from the patient heterozygous for p.Cys2693Tyr. The majority of the cells showed few and relatively short WPB concomitant with retention of VWF in the ER, while a minority of cells showed completely normal WPB formation (Figure 2F-F'). This phenomenon suggests that not all cells express the mutant (p.Cys2693Tyr) protein. Alternatively, all the mutant protein and the heterogeneous dimers (with WT-VWF) were degraded within the ER in the minor cell population. Consequently, only WT-VWF dimers were transported and gave rise to normal elongated WPB.

The advantage of using BOECs over HUVECs or other primary endothelial cells is the much easier access to patients with different VWF mutations. HUVECs have only been extracted from the cords of sporadic VWD patients [18-22]. Other endothelial sources are even more difficult to access. In contrast, BOECs can, in principle, be obtained from the peripheral blood of almost any consenting subject. We have so far isolated BOECs from more than 20 subjects, and only two of them failed to yield BOECs. Importantly, except for the BOECs derived from the patient heterozygous for p.Tyr1584Cys, the other 9 subjects' BOECs reported in this study were obtained from the first isolation. Therefore, in practice, the isolation approach described here is simple and can be routinely applied with a high rate of success.

The main source of plasma VWF is the vascular endothelial cell in which VWF is stored in WPB [4]. Alterations in VWF structure may impair biogenesis of WPB and consequently impair secretion of VWF [11,12,45]. Baseline plasma VWF levels may be mainly determined by basal secretion of VWF from WPB [5]. Consistent with the severity of plasma VWF deficiency in the patients, basal secretion of VWF was normal or slightly reduced for BOECs derived from the two mild type 1 VWD patients (p.Tyr1584Cys and p.Cys2693Tyr), while it was strongly reduced for BOECs derived from the two moderate type 1 VWD patients (p.Ser1285Pro and p.Leu1307Pro). The reduced basal secretion may be caused by retention of VWF in the ER and the resultant reduction in WPB formation, reduced VWF production, or the combination of the two. The strikingly low production of VWF in BOECs derived from the patients heterozygous for p.Ser1285Pro and p.Leu1307Pro suggested that a dominant-negative effect of these two mutations led to gross intracellular degradation of VWF as described for VWF mutation p.Cys1149Arg [36]. This intracellular degradation of VWF is probably induced by retention of VWF in the ER (Figure 2) [36,46]. Surprisingly, the basal secretion of VWF from BOECs with the type 2N mutation p.Arg854Gln appeared slightly reduced compared with

BOECs derived from healthy donors. This suggests that the production and basal secretion of VWF from BOECs should be interpreted with caution. Actually, both total production and basal secretion of VWF varied between passages of BOECs cultures, and between BOEC clones derived from the same individuals. In addition, total VWF production, basal secretion of VWF and cell proliferation also varied between BOECs derived from different healthy donors. Due to these limitations, mild quantitative defects caused by mutations such as p.Tyr1584Cys may go unnoticed, whereas some VWF mutations with a functional defect, but normal antigen levels, such as p.Arg854Gln, might appear to reduce VWF production and basal secretion, all depending on the BOECs culture conditions. To minimize variation caused by those factors, we chose to assess production and basal secretion at the same culture passage for all BOECs. This variability might also be overcome by isolating BOECs from a larger number of healthy controls, but for specific mutations this will not be feasible as the number of available patients carrying specific mutations is usually small.

We analyzed the intracellular storage and regulated secretion of VWF in several VWD patients. Even in patients mainly characterized by quantitative deficiency in VWF (mutations p.Ser1285Pro, p.Leu1307Pro and p.Cys2693Tyr) we identified abnormalities in the biogenesis of WPB in the BOECs. BOECs with VWF mutations p.Arg854Gln (type 2N) or p.Tyr1584Cys (type 1) showed normal storage of VWF in elongated WPB, similar to what is seen in healthy BOECs. In line with previous findings [11,12,45], impaired storage of WPB was probably caused by the increased retention of VWF in the ER rather than by a decrease in production of VWF. Indeed, BOECs with p.Arg854Gln stored VWF properly even though the total VWF production was much lower than that of BOECs with p.Cys2693Tyr in which abnormal WPB dominated. Impaired storage of VWF in WPB resulted in defective exocytosis of WPB. Consequently, the formation of VWF strings was also impaired (Figure 6). In particular, VWF strings formed by BOECs heterozygous for p.Ser1285Pro and p.Leu1307Pro were much less and shorter. We postulated that the response to stimulation of WPB in the two BOECs was strongly reduced because of the abnormal WPB formation (Figure 5). Another explanation could be that the diminished storage of VWF in these two BOECs led to less VWF string formation, and consequently to insufficient self-association of VWF strings which resulted in shorter strings. Similar mechanisms might occur to BOECs with p.Cys2693Tyr but to a lesser extent. No apparent defects in VWF strings were observed for BOECs with p.Arg854Gln and p.Tyr1584Cys. The defects in VWF

string formation by different BOECs were confirmed respectively by several WPB secretagogues, including PMA, histamine and epinephrine via distinct signaling pathways (Figure 6, 7 and Supplemental Figure S3). More importantly, defects in formation of VWF strings seem well correlated with the phenotypic severity of the patients from whom BOECs were derived.

In conclusion, BOECs provide an easily accessible *bona fide* cell model to analyze the properties of VWF mutations *in vitro*. BOECs were particularly useful to study aspects of intracellular storage, regulated secretion and string formation of VWF. They also provided an optimal model to investigate possible dominant-negative effects that may be present in the heterozygous state. Furthermore, this endothelial cell model can be used to examine the response to several WPB secretagogues because the relative secretion of VWFpp was relatively constant between cell passages and clones.

Acknowledgement

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Supplemental data

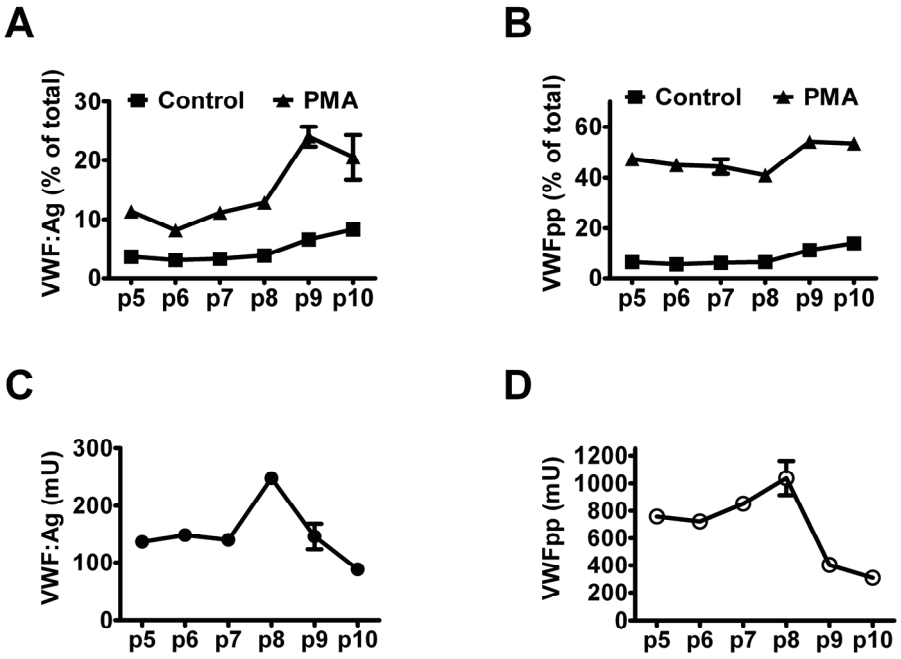

Figure S1. Regulated secretion of VWF and VWFpp from BOECs derived from a VWD patient. BOECs derived from a VWD patient heterozygous for mutation p.Tyr1584Cys were cultured in 12-well plates until confluent. After rinsing with HBSS, cells were incubated for 60 minutes without (Control) or with 160 nM PMA (PMA) at 37 °C. Regulated secretion of VWF (A) and VWFpp (B) was determined for BOECs at given passages. In panels C and D, the Y-axis indicates the total production (medium plus lysate) of VWF or VWFpp by approximate $1x10^5$ cells. Mean ± SEM indicate variation between triplicates in one representative experiment.

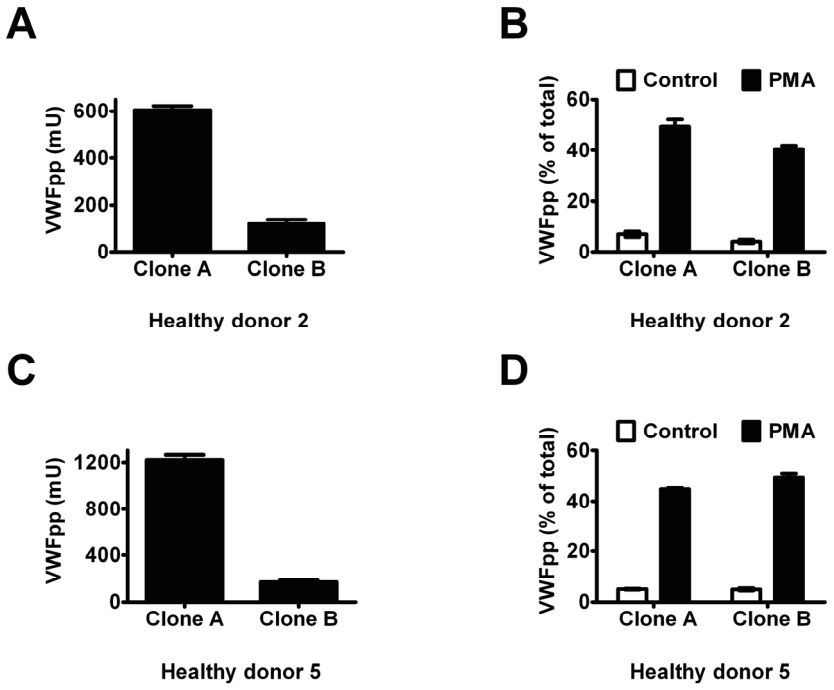

Figure S2. Secretion of VWFpp from BOECs, comparison between clones. BOECs derived from different clones were cultured in 12-well plates at passage 6 (A-B) or passage 5 (C-D). Confluent cell layers were incubated for 60 minutes at 37 °C in the absence (Control) or presence of 160 nM PMA (PMA). Each bar represents the total production (medium plus lysate) of VWFpp by approximate $1x10^5$ cells in panels A and C or the percentage of secreted VWFpp (a fraction of total, i.e. VWFpp in the medium plus VWFpp in the cell lysate) in panels B and D. Mean ± SEM indicate variation between triplicates in one representative experiment. BOECs derived from two healthy donors (healthy donor 2 and 5) are shown as examples.

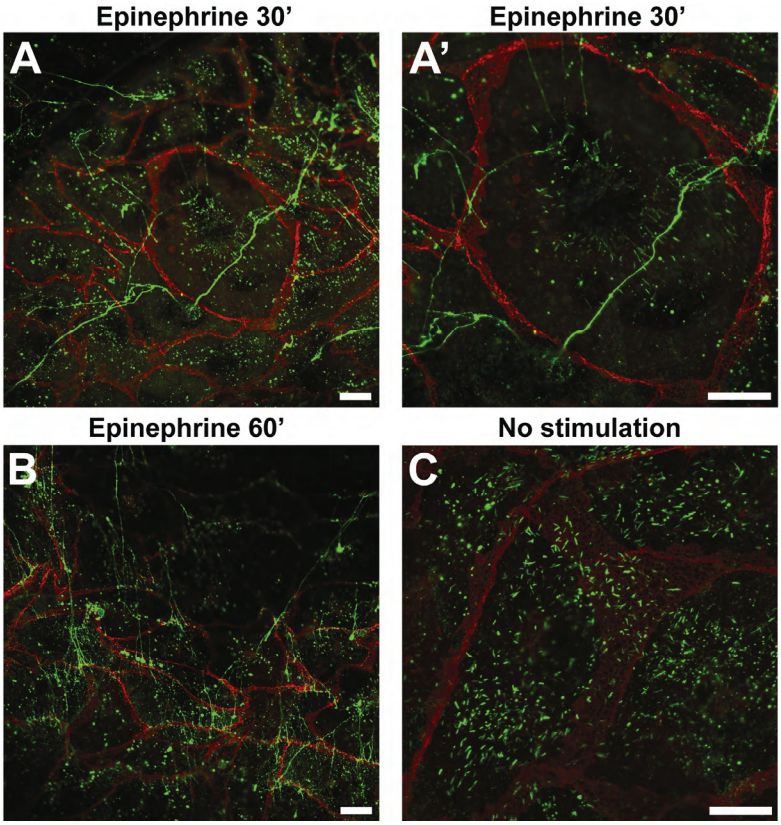

Figure S3. Exocytosis and movement of WPB upon stimulation with epinephrine. BOECs derived from a healthy donor were stimulated for 30 minutes (A and A') or 60 minutes (B) with 10 µM epinephrine plus 100 µM IBMX at 37 °C. In panel C, normal distribution of WPB in BOECs that did not undergo stimulation was shown as comparison. Cells were fixed and stained for VWF (green) and β-catenin (red). As shown by panel A most of WPB disappeared upon short stimulation with epinephrine and VWF strings were formed on the cell surface. The remaining WPB (elongated green organelles) clustered in the perinuclear area (a cell in panel A was closed up shown in panel A'). After 60 minutes stimulation, almost all WPB disappeared. Scale bars represent 20 µm. All images were taken by Leica SL confocal laser scanning microscopy with a 63X/1.40 NA oil objective.