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NONTRADITIONAL ROLES FOR THE HEMOSTATIC SYSTEM IN THE VESSEL WALL

von Willebrand factor regulation of blood vessel formation

Anna M. Randi,[1] Koval E. Smith,[1] and Giancarlo Castaman[2]

[1]Vascular Sciences, Imperial Centre for Translational and Experimental Medicine, National Heart and Lung Institute, Imperial College London, London, United Kingdom; and [2]Center for Bleeding Disorders and Coagulation, Department of Oncology, Careggi University Hospital, Florence, Italy

Several important physiological processes, from permeability to inflammation to hemostasis, take place at the vessel wall and are regulated by endothelial cells (ECs). Thus, proteins that have been identified as regulators of one process are increasingly found to be involved in other vascular functions. Such is the case for von Willebrand factor (VWF), a large glycoprotein best known for its critical role in hemostasis. In vitro and in vivo studies have shown that lack of VWF causes enhanced vascularization, both constitutively and following ischemia. This evidence is supported by studies on blood outgrowth EC (BOEC) from patients with lack of VWF synthesis (type 3 von Willebrand disease [VWD]). The molecular pathways are likely to involve VWF binding partners, such as integrin αvβ3, and components of Weibel-Palade bodies, such as angiopoietin-2 and galectin-3, whose storage is regulated by VWF; these converge on the master regulator of angiogenesis and endothelial homeostasis, vascular endothelial growth factor signaling. Recent studies suggest that the roles of VWF may be tissue specific. The ability of VWF to regulate angiogenesis has clinical implications for a subset of VWD patients with severe, intractable gastrointestinal bleeding resulting from vascular malformations. In this article, we review the evidence showing that VWF is involved in blood vessel formation, discuss the role of VWF high-molecular-weight multimers in regulating angiogenesis, and review the value of studies on BOEC in developing a precision medicine approach to validate novel treatments for angiodysplasia in congenital VWD and acquired von Willebrand syndrome. (*Blood*. 2018;132(2):132-140)

Introduction

von Willebrand factor (VWF) is a large glycoprotein that mediates platelet adhesion to the subendothelium during vascular injury and stabilizes coagulation factor VIII (FVIII).[1] The importance of VWF in hemostasis is illustrated by the fact that its deficiency and/or abnormality causes von Willebrand disease (VWD), the most frequent inherited bleeding disorder. Over the past 2 decades, other roles for VWF in the vasculature have been identified, including inflammation, permeability, and angiogenesis (reviewed in Rauch et al[2]). The function of VWF is linked to its interaction with multiple cellular and extracellular proteins and to its ability to coordinate the formation of Weibel-Palade bodies (WPBs). The relevance of these new roles of VWF to patients with VWD is still to be defined. The ability of VWF to regulate angiogenesis, however, is connected with gastrointestinal (GI) vascular malformations observed in some patients with VWD and acquired von Willebrand syndrome (AVWS), which can cause severe bleeding. In this article, we will review the evidence supporting a role for VWF in angiogenesis and the clinical implications of these findings.

VWF and the vascular endothelium

VWF structure-function studies and binding partners

In EC, VWF is constitutively synthesized and stored in WPB.[3,4] Plasma VWF derives almost entirely from constitutive endothelial secretion[5,6]; VWF released into the subendothelium is involved in EC adhesion and binding to extracellular matrix.[7] The *vwf* sequence on chromosome 12 codes for a pro-polypeptide of 2813 amino acids, of which 2050 form the mature peptide.[8,9] VWF is synthesized as a monomer of ~220 kD containing multiple domains[10]; some domains mediate binding to a wide array of proteins. The number of reported VWF binding partners is increasing rapidly (Table 1). Many interactions, including binding to platelet GPIb and collagen, are localized in the A domains. The binding partners' profile for VWF is likely to vary depending on its location (plasma vs vessel wall vs intracellular compartments), the local microenvironment, and physiological or pathological cues. Experimental data for each ligand will be required to build a functional map of VWF interactions.

Another key determinant of VWF function is its multimeric structure. The processes of VWF biosynthesis and multimerization have been reviewed elsewhere.[11,12] The entire spectrum of circulating VWF species ranges from single dimer to multimers made of up to 20 dimers,[13] with high-molecular-weight multimers (HMWMs) the most hemostatically active. Several lines of evidence suggest that VWF multimerization is also important for VWF regulation of angiogenesis (see "VWF and WPB: the endothelial storage cupboard"). Control of multimerization is carried out by a specific plasma protease, called a disintegrin-like and metalloprotease with thrombospondin-type 1 repeats, member-13 (ADAMTS-13). This activity is regulated by high-shear stress forces, which stretch VWF in the circulation thus exposing the cleavage site for ADAMTS-13 in the A2 domain.[14,15] The importance of ADAMTS-13 in regulating

Table 1. VWF binding partners

Family	Protein	VWF interacting domain	Reference
Plasma proteins	FVIII	D′D3	100
	ADAMTS13	A1-A2-A3	101
	Fibrin	C1-C6	102
	Complement components	C1-C2	103
Platelet receptors	GP Ib	A1	104
	GP IIbIIIa	C4	105
	P-selectin	D′-D3	106
EC receptors	Integrin αvβ3	C4	7,86
	P-selectin	D′-D3	106
	Choline transporter like protein-2	A1	107
	CLEC4M	—	108
VSMC receptors	Integrin αvβ3	C4	80
Leukocyte receptors	β2 integrins	D′-D3, A1-A2-A3	109
	PSGL-1	Glycans	109
	Siglec-5	—	110
	LRP1	—	111
ECM proteins	Collagen I	A3	112
	Collagen III	A3	112
	Collagen VI	A1	113
	Thrombospondin	A3	114
	Heparin	A1	115
Others (plasma and/or cellular)	Histones	A1	116
	DNA/NETs	A1	117
	Angiopoietin-2	A1	118
	Interleukin-8	—	119
	Galectin 1 and 3	Glycans	97
	Osteoprotegerin	A1	120
	Multimerin 1	A1 A2 A3	121

ECM, extracellular matrix; NET, neutrophil extracellular trap.

hemostasis is demonstrated by the fact that its deficiency (by genetic mutations in ADAMTS-13 or inhibitory antibodies) causes the rare disorder thrombotic thrombocytopenic purpura, in which ultralarge VWF HMWM, similar to those present in WPB, are uncleaved and induce platelet aggregation and thrombotic microangiopathy.[16]

VWF and WPB: the endothelial storage cupboard

In EC, HMWM of VWF are stored in WPB and released upon stimulation, whereas the lower MWM are released constitutively.[5] Detailed studies have shown that VWF directs the biogenesis of WPB. Transfection of VWF pro-peptide or recombinant VWF into non-EC induces formation of WPB structures.[17-20] Conversely, lack of VWF expression either in small interfering RNA (siRNA)-treated human umbilical vein endothelial cells or in blood outgrowth EC (BOEC) from type 3 (null) VWD results in loss of WPB.[21] Studies on naturally occurring VWF mutations in HEK-293 cells and on BOEC from patients with type 1 and type 2 VWD showed that decrease and/or abnormal processing of VWF can also affect WPB formation.[22-24]

Mass spectrometry studies have recently characterized the content of WPB.[25,26] Together with VWF, WPB store proteins that regulate vascular processes such as angiogenesis and inflammation, including osteoprotegerin, galectin-3 (Gal-3), P-selectin, interleukin-8, and angiopoietin-2 (Ang-2).[4,27] Interestingly, not all WPB contain all components; indeed, some WPB proteins, such as Ang-2 and P-selectin, appear to be mutually exclusive.[28] Moreover, WPB distribution appears heterogeneous among EC

within the same vascular bed.[20,29] Early studies investigating the distribution of WPB in the vasculature found lower WPB numbers in microvessels,[30] with fewer WPB in arteriolar endothelium compared with capillaries.[31]

VWF heterogeneity in EC and tissues

VWF expression has been used for decades as a marker of the vascular endothelium. However, several studies have reported heterogeneous levels of VWF in EC from different tissues and in arterial vs venous vs capillary endothelium.[32-36] Early studies, mostly in nonhuman tissues, showed that VWF messenger RNA is expressed at higher levels in the venous endothelium compared with arteries and arterioles. Interestingly, the different VWF levels in EC from different sources are maintained in cultured EC.[35] Elegant studies by Aird et al demonstrated the influence of the microenvironment on endothelial VWF expression.[37] Some transcriptional activators/repressors and epigenetic mechanisms that control tissue-specific expression of VWF have been investigated.[38] Remarkably, VWF expression can also vary between EC within the same tissue.[39-42] The heterogeneity of expression of VWF is likely to affect its role in a tissue-specific manner, as suggested by experimental data (see "VWF as a marker of angiogenesis").

VWF as a marker of angiogenesis

In 1971, Zimmerman first reported immunological detection of the protein known today as VWF, called FVIII-related antigen at the time.[43] The overlap between VWF and FVIII antigen terminology remained for several years; hence, early publications on VWF expression in the vasculature refer to FVIII-related antigen or antihemophilic factor. In 1972, Hoyer et al reported that an antibody to the "antihemophilic factor" detected a protein expressed in the endothelium.[44] Since then, hundreds of studies have used VWF as a marker of EC, because of its selective endothelial expression, combined with high abundance and good specific antibodies. VWF expression has been used extensively to quantify angiogenesis in a variety of tumors.[45,46] However, VWF endothelial expression itself may be regulated in tumor endothelium. Zanetta et al reported upregulation of VWF expression by angiogenic factors vascular endothelial growth factor (VEGF) and fibroblast growth factor-2,[47] which are highly present in the tumor microenvironment. Conversely, anti-VEGF treatment with bevacizumab has been shown to decrease VWF plasma levels.[48] Thus, at least in some tumors, VWF expression may not only reflect the increase in microvasculature, but could also be a consequence of specific regulation of its expression.

VWF as a regulator of angiogenesis: clinical evidence

Decrease or dysfunction of VWF causes VWD. The disease is highly heterogeneous, with a complex classification[49] that can be simplified to identify 3 main types: type 1, in which VWF plasma levels are decreased; type 2, resulting from dysfunctional VWF; and type 3, with virtual absence of plasma VWF. VWD can also be acquired (AVWS) in circumstances in which circulating VWF is dysfunctional or reduced from a variety of pathogenic mechanisms including autoantibodies and mechanical-induced unfolding followed by proteolytic cleavage.[50] Bleeding is the main clinical manifestation in both congenital and acquired VWD; however, a connection between VWF and vascular abnormalities has been known for a long time. Early descriptions of VWD in the 1930s included abnormalities of the Rumpel-Leede test, suggesting a vascular defect.[51] An association between VWD and hereditary hemorrhagic telangiectasia, characterized by arterial-venous malformations and bleeding,[52] was also reported; however, a clear common pathogenetic mechanism shared by the 2 disorders has never been demonstrated. Vascular abnormalities have been reported in patients with congenital VWD: a systematic evaluation of the microcirculation in 100 patients with VWD, using intravital capillary microscopy (capillaroscopy), revealed morphological changes of the nail fold capillaries, with increased dilatation, microscopic bleeding, and torquation (dysplasia).[53] Thus, lack or dysfunction of VWF correlates with systemic malformations of mucosal vasculature.

Vascular malformations and angiodysplasia in VWD and AVWS

The most significant evidence of the link between VWD and vascular malformations is angiodysplasia of the GI tract. Endoscopies carried out because of bleeding of the GI tract show that angiodysplasia is a major cause of digestive tract bleeding and is commonly observed in elderly people, with incidences ranging from 2.6% to 6.2%.[54,55] In VWD patients, GI bleeding often occurs at a younger age. Interestingly, GI bleeding potentially associated to angiodysplasia occurs mainly in VWD patients lacking VWF HMWM.[56-58] In a retrospective study on 4503 VWD patients, the incidence of angiodysplasia was 0% in type 1, 2% in type 2, and 4.5% in type 3 VWD.[56] Bleeding from angiodysplasia occurs mainly in types 2A, 2B, and 3 VWD, characterized by the lack of VWF HMWM.[56-58] A multicenter retrospective study found that the risk of GI bleeding in patients with type 1 VWD was only marginally increased compared with the general population.[59] A large prospective study comparing type 2M and 2A VWD patients, with similar FVIII and VWF levels, showed a higher bleeding risk in type 2A patients because of a high prevalence of GI bleeding.[60] Interestingly, no GI bleeding was observed in a 7-year prospective study on patients with VWD Vicenza, a clearance defect causing lower plasma FVIII and VWF than VWD type 2A patients, but with ultralarge VWF multimers.[61] Thus, clinical evidence supports the important role of HMWM in GI bleeding. However, a retrospective study within the VWD Prophylaxis Network of 48 VWD patients with GI bleeding found that most types of VWD (types 1, 2A, 2B, 2M, and 3) were represented, including 9 patients with type 1 VWD (18.7%), 4 of which had documented angiodysplasia. In line with previous reports, the majority (60.4%) of patients with documented angiodysplasia had type 2A or 3 VWD.[62] These studies suggest that the lower the VWF activity, the more likely is development of abnormal GI vessels. The study also highlighted the complexity of diagnosing angiodysplasia in patients with GI bleeding, because in >60% of patients, the cause of bleeding remained unexplained, despite adequate investigation.

The importance of HMWM in preventing GI bleeding has also been suggested in AVWS.[63] Occurrences of GI bleeding and AVWS have been described mainly in 2 specific acquired conditions. Heyde syndrome is a rare condition that occurs in 3% of patients with aortic stenosis and is associated with GI bleeding believed to be caused by the loss of VWF HMWM.[44,64,65] In some of these patients, HMWM are lacking as a result of high-shear

Table 2. Therapeutic approaches to GI bleeding

Agent	No. patients	VWD type	Outcome	Reference
Octreotide	2	1	Successful	122
		2A		
Thalidomide	1	2B	Successful, previous failure with octreotide	123
	1	2B	Successful, several drugs associated	124
	5	NA	No response	62
HD atorvastatin	1	1	Successful, previous failure with octreotide and thalidomide	125
	1	2A	Successful, previous failure with thalidomide	126
Tamoxifen	2	3	Successful	127

HD, high dose; NA, not available.

stress exerted by the stenotic valve, with increased stretching of the VWF molecule and increased susceptibility to ADAMTS-13.[66] In patients with AVWS associated with left ventricular assisted devices (LVADs), GI bleeding has also been linked to the presence of angiodysplasia.[67] Despite the widespread finding of AVWS, not every patient with an LVAD develops major bleeding complications, suggesting that other key determinants of bleeding may vary in this population. The prevalence of angiodysplasia in LVAD patients is not known because the invasive techniques used for diagnosis, namely endoscopy, carry a particularly high risk of complications in these patients. This highlights the need for a noninvasive imaging tool that could help predict bleeding risk and stratify patients for management and outcome.

Several reports have described GI bleeding and angiodysplasia in lymphoproliferative disorders, including monoclonal gammopathy of uncertain significance, chronic lymphocytic leukemia, and multiple myeloma.[68] In these disorders, the reduction of VWF and HMWM is attributable either to adsorption of VWF onto malignant cells or to increased clearance of the complex between monoclonal immunoglobulins and VWF.[69]

Treatment of angiodysplasia-associated GI bleeding in VWD

The mechanism responsible for angiodysplastic malformations in VWD is unknown; hence, treatment of bleeding associated with angiodysplasia can be very difficult. A few patients may be treated by surgical or endoscopic approaches when the lesions are limited in extension and well identified. Although patients may initially respond well to treatment with VWF-FVIII concentrates, bleeding often recurs and prophylactic regimes have been tried with partial success.[70] The prophylactic treatment may be very costly and the recurrence of bleeding affects significantly the quality of life of these patients. Alternative pharmacological approaches have been anecdotally tried in a few patients using drugs with antiangiogenic action (Table 2).[62,71] Thalidomide is an antiangiogenic drug with anti-inflammatory and immunomodulatory effects.[72] The antiangiogenic effects may be linked to its ability to decrease expression of VEGF.[73] Atorvastatin exerts dose-dependent effects on angiogenesis: at high doses, it has been shown to inhibit angiogenesis through effects on endothelial proliferation and survival.[74] Octreotide is typically used for the control of hemorrhage from esophageal varices and in patients with angiodysplasia not associated with VWD because it induces a reduction in splanchnic and portal blood flow.[75] In VWD, these drugs have shown inconsistent results in controlling GI bleeding; because of the rarity of the disorder, properly designed clinical studies on a sufficient number of patients are difficult to perform.

In AVWS, surgical correction of valve stenosis is accompanied by the restoration of a normal multimeric pattern and the abolishment of bleeding complications.[65] There have been reports of GI bleeding and angiodysplasia in AVWS patients with monoclonal gammopathy of uncertain significance, resistant to desmopressin and VWF/VIII concentrates, which responded successfully to prophylaxis with intravenous immunoglobulins, with normalization of FVIII and VWF levels and multimeric pattern.[68,76,77] The challenges of treating GI bleeding in VWD and AWVS patients highlights the need to identify the molecular pathways that may provide novel therapeutic targets.

VWF as a regulator of angiogenesis: experimental evidence

A few years ago, direct evidence for VWF's role in the control of angiogenesis was reported.[21] In vitro studies on EC showed that inhibition of VWF expression using siRNA resulted in increased proliferation, migration, and in vitro angiogenesis.[21] A similar overall pattern was found in BOEC from patients with VWD,[21,78,79] although significant differences in the cellular phenotypes have been observed depending on different molecular defect. Conversely, plasma-derived VWF was shown to inhibit endothelial tube formation in an in vitro model of angiogenesis.[21] In VWF-deficient mice, angiogenesis and vascular density were increased in vivo,[21] whereas recruitment of vascular smooth muscle cells (VSMCs), a sign of arterial maturation, was delayed in the developing retinal vasculature.[80] Recently, Xu et al reported enhanced angiogenesis in the brain of VWF-deficient mice in response to hypoxia.[81] Interestingly, this study also investigated the angiogenic response to ischemia in ADAMTS-13–deficient mice, where VWF is not cleaved and hence levels are increased. In these mice, angiogenesis was decreased and was normalized by inhibition of VWF

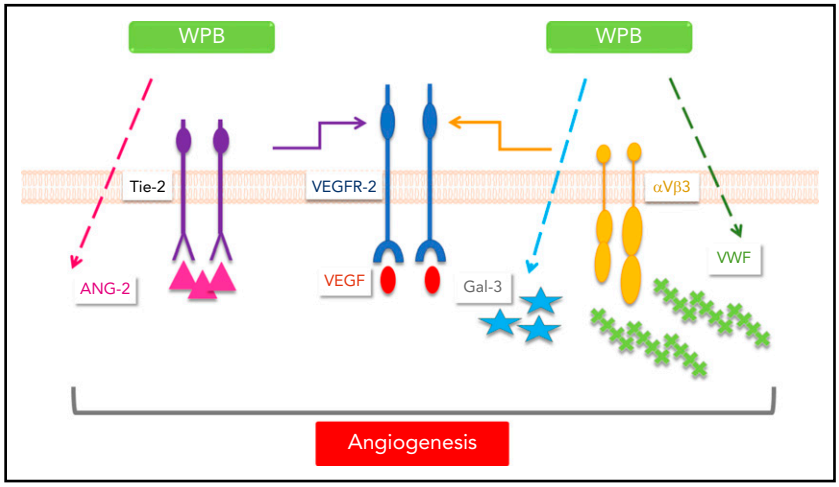

Figure 1. VWF regulation of angiogenesis through multiple pathways: model. A model for multiple pathways likely to be involved in VWF regulation of angiogenesis. In EC, VWF is essential for the formation of WPB, organelles that store the growth factor Ang-2. Loss of intracellular VWF results in increased release of Ang-2 from WPB; Ang-2 binding to its receptor Tie-2 can synergize with VEGFR-2 signaling to destabilize blood vessels and promote angiogenesis. Thus loss of VWF could enhance angiogenesis via an Ang-2/Tie-2/VEGFR-2 pathway. Moreover, VWF released from WPB interacts with the adhesion receptor integrin αvβ3 and stabilizes its expression on the cell surface. In selected conditions, αvβ3 integrin is able to quench VEGFR-2 activity and downstream signaling, thus exerting a repressive effect on angiogenesis. Loss of VWF in EC results in decreased αvβ3 expression, which may cause enhanced VEGFR-2 signaling. Enhanced, deregulated VEGFR-2 signaling has been shown to cause dysfunctional angiogenesis leading to dysplastic blood vessels, similar to those described in angiodysplasia. Finally, WPB also store the carbohydrate-binding protein Gal-3. Gal-3 promotes angiogenesis through pathways that involve both αvβ3 and VEGFR-2; levels of Gal-3 are increased in brain microvasculature of VWF-deficient mice. Thus, VWF is likely to affect multiple angiogenic pathways, both as an extracellular ligand and because of its ability to control storage and possibly expression of endothelial proteins. VEGFR-2, VEGF receptor-2.

expression or activity, demonstrating that the effect of ADAMTS-13 on angiogenesis is dependent on VWF. The conclusion from these studies is that VWF is involved in blood vessel formation, with a predominantly inhibitory role. However, contrary to these reports, a recent study by de Vries et al[82] found impaired arteriogenesis and angiogenesis in VWF-deficient mice following ligation of the femoral artery. These findings highlight the complexity of the role of VWF in modulating blood vessel formation and support the hypothesis of a tissue- and stimulus-specific function.

It is important to stress that the role exerted by VWF in the modulation of blood vessel formation is mild and clearly not essential for embryonic development, given that severe VWF deficiency occurs in patients who can reach adulthood, and in many cases old age, and no apparent severe developmental issues are associated with lack of VWF in patients or in animal models. However, the enhanced vasculature in the ear[21] and brain[75] in VWF-deficient mice, and the presence of dysplastic vessels in the nail bed of VWD patients,[53] point to a mild effect of VWF on vascular development.

VWF, angiodysplasia, and angiogenesis: molecular pathways

Given the multiple binding partners of VWF (Table 1), there are many potential mechanisms through which VWF may influence blood vessel formation. The ability of VWF to control the formation of WPB offers another possible route to the control of angiogenic modulators. So far, the evidence points to VWF modulating angiogenesis through a network of pathways, schematically summarized in Figure 1. These pathways have been recently reviewed elsewhere[83]; here, we will briefly summarize the evidence, highlight recent findings, and discuss their possible relative importance.

VEGFR-2 signaling

In vitro studies implicate VEGFR-2 signaling in the phenotypes of VWF-deficient EC.[21] This has now been confirmed in vivo since enhanced VEGFR-2 phosphorylation was found in the microvasculature of VWF-deficient mice. Many studies have shown that excessive, dysregulated VEGF signaling causes formation of unstable, fragile, and leaky vessels,[84] similar to angiodysplastic lesions; indeed, a role for increased VEGF has been proposed in angiodysplasia.[73,85] This is an attractive hypothesis that speculates that dysregulated VEGFR-2 signaling could lead to the development of abnormal vasculature as observed in VWD patients. How VWF modulates VEGFR-2 signaling is unclear. The original hypothesis of a role for integrin αvβ3, a VWF ligand[86] that controls activity of VEGFR-2,[87] still requires validation.

VWF binds to αvβ3 via its arg-gly-asp sequence in the C-terminal region.[86] Surprisingly, very little is known about the signaling events that follow VWF binding to αvβ3 on EC. Interaction of other αvβ3 ligands, such as vitronectin, is known to activate signal transduction via complex formation with adaptor proteins and kinases including FAK and Src-family kinases at focal adhesion complexes.[88] Whether VWF also affects these pathways is currently unknown. VWF can also interact with αvβ3 on VSMC, and Scheppke et al implicated this pathway in arterial maturation during vascular development.[80]

WPB proteins: Ang-2 and Gal-3

VWF drives the formation of WPB, the endothelial storage organelles that contain multiple proteins, including the angiogenesis regulator Ang-2.[28] Ang-2 is part of the angiopoietins/tie-2 pathway, a crucial system regulating vascular homeostasis and angiogenesis.[89] Ang-2 has been shown to destabilize blood vessels and synergize with VEGF to promote angiogenesis.[90,91] In vitro studies on VWF-deficient EC (siRNA-treated or BOEC from type 3 VWD) show that VWF regulates the endothelial

storage and release of Ang-2.[21,92] Interestingly, this is not a generalized effect on all WPB proteins, because interleukin-8 release is not regulated by VWF (K.E.S. and A.M.R., unpublished data). VWF also controls Ang-2 synthesis: messenger RNA levels of Ang-2 are increased in EC treated with VWF siRNA,[21] in BOEC from type 3 VWD patients,[79,92] and in EC from hearts of VWF-deficient mice.[42] Interestingly, the regulation of Ang-2 levels by VWF appears to be tissue-specific, because it was not observed in the kidney or liver of VWF-deficient mice.[42] Increased Ang-2 levels in mouse hearts was accompanied by significant microvascular damage of heart capillaries and abnormal cardiac function. These data raise the intriguing possibility that VWF's tissue-specific control of Ang-2 storage and expression may result in vascular and ultimately organ dysfunction, possibly by affecting VEGFR-2 signaling. This pathway is not only a major regulator of blood vessels homeostasis, but has also been shown to influence cardiomyocyte survival,[93] myocardial blood flow, and hemodynamics,[94] indicating another possible mechanism underlying the abnormal cardiac function described in VWF-deficient mice. The relevance of these pathways in VWD patients remains to be established.

Another WPB molecule recently implicated in VWF-dependent control of angiogenesis is Gal-3, a carbohydrate-binding protein that promotes angiogenesis.[95] Gal-3 inhibitors block VEGF-mediated angiogenesis in vitro.[96] It also binds to both VEGFR2, promoting its phosphorylation, and to integrin αvβ3; its interaction with the integrin is essential for its proangiogenic activity.[96] Gal-3 also binds VWF[97]; this suggest a complex network of pathways that can modulate angiogenesis. In VWF-deficient mice, Gal-3 levels were increased in the brain microvasculature after stroke[81]; thus, it is possible that, at least in this tissue, raised Gal-3 levels contribute to the enhanced angiogenesis. Gal-3 inhibitors, already in clinical trials and exhibiting a good safety profile (www.clinicaltrials.gov: NCT01899859), may represent an interesting future option for treatment of GI bleeding.

Angiogenesis studies in VWD patients: circulating markers

Enhanced expression of VEGF[85] and Ang-2[98] has been described in regions of the colon affected by angiodysplasia and in plasma from non-VWD patients with sporadic bowel angiodysplasia. Recent studies from Groeneveld et al on plasma from a cohort of VWD patients comprising 395 type 1, 239 type 2, and 21 type 3 revealed that median levels of Ang-2 were significantly reduced, whereas levels of Ang-1 and VEGF were increased compared with controls.[99] Because platelets are a large source of Ang-1, it is possible that platelet activation might have contributed to the enhanced levels of Ang-1 in plasma samples. In patients who experienced GI bleeding resulting from angiodysplasia, there was a significant increase in Gal-3 levels compared with patients with bleeding from different sites.[99] There was also a trend toward increased Ang-2 levels and toward decreased Ang-1 levels. The balance between these growth factors (and their relationship with the VEGF pathway) is crucial to the formation and maintenance of healthy vasculature[89]; therefore, it is possible that an Ang-1/Ang-2 imbalance could contribute to aberrant vascularization seen in these vascular lesions.

Angiogenesis studies in VWD patients: BOEC

Access to EC from VWD patients via the isolation of BOEC from peripheral blood represents a game changer in the potential to understand the vascular consequences of loss or dysfunction of VWF. Based on the models discussed here, it is clear that different defects causing VWD may result in different cellular phenotypes. Thus, one could predict that in VWD patients with disrupted endothelial WPB, such as type 3 and severe type 1 patients, the Ang-2 pathway may be predominant. However, in patients with dysfunctional VWF, such as type 2 VWD, WPB appear mostly normal; in these patients, the interaction of VWF with the EC surface and/or with extracellular protein may contribute to the angiogenic phenotype. To date, 3 reports have investigated the angiogenic phenotypes of BOEC from VWD.[21,78,79] In 2011, Starke et al studied the in vitro angiogenic potential of BOEC isolated from 9 VWD patients (types 1 and 2) and found overall significantly enhanced in vitro angiogenic profiles, in line with VWF-deficient human umbilical vein endothelial cells.[21] The following studies have identified distinct in vitro angiogenesis defects in BOEC, based on clinical classification. Groeneveld et al showed a decrease in directional migration in BOEC from type 1, but not type 2 VWD, compared with controls.[78] This study also demonstrated that the increased angiogenic phenotype observed in early passages of BOEC from type 1, 2, and 3 patients was lost at later passages, highlighting one of the many technical issues with this approach (see "BOEC methodological issues and future perspectives"). Selvam et al reported heterogeneous phenotypes in BOEC from 5 patients with type 3 VWD and variable levels of VWF synthesis.[79] These studies show a range of abnormalities, at times unexpected, with no overall consistent picture, in line with the complexity and heterogeneity of this disease. They also highlight the great potential of using VWD BOEC to dissect the pathways through which VWF controls angiogenesis. An interesting question is whether BOEC represent a valid model for the investigation of cellular mechanisms underlying VWD, given the tissue-specific profiles observed in VWF-deficient mice. Because of their progenitor state, BOEC could recapitulate a cellular state not influenced by the tissue microenvironment. Moreover, BOEC are ideal for coculture and 3-dimensional models mimicking different vascular beds. Such models, currently being developed in several laboratories, will significantly change the way we investigate vascular function and disease.

BOEC methodological issues and future perspectives

Optimization and standardization of protocols for isolation, in vitro expansion, and analysis are required to allow a better interpretation of the results obtained with BOEC. Some key technical points have emerged from these early studies. Crucially, BOEC isolation currently requires a fresh blood sample, which significantly limits the application of the method. Careful phenotypic characterization of the cells before functional assays is required. Also, differences in proliferation rates will result in different population doublings for cells at the same passage number, the convention used to standardize the "age" of cells in culture. This may be a problem for BOEC cells with a high replicative potential, such as VWF-deficient cells.[21,78] These limitations need to be overcome, given the significant value that cellular studies could add to the understanding of this disease. A major application of BOEC studies will be to define the relative importance of

the different molecular mechanisms described previously, and more to come, in the regulation of blood vessel formation. More studies on VWD BOEC will allow the design of personalized treatment of angiodysplasia and intractable GI bleeding in VWD.

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ORCID profile: A.M.R., 0000-0002-0729-211X.

Correspondence: Anna M. Randi, National Heart and Lung Institute, Hammersmith Hospital, Imperial College London, London, United Kingdom; e-mail: a.randi@imperial.ac.uk.

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