The recent discovery that von Willebrand factor (VWF) regulates blood vessel formation has opened a novel perspective on the function of this complex protein. VWF was discovered as a key component of haemostasis, capturing platelets at sites of endothelial damage and synthesized in megakaryocytes and endothelial cells (EC). In recent years, novel functions and binding partners have been identified for VWF. The finding that loss of VWF in EC results in enhanced, possibly dysfunctional angiogenesis is consistent with the clinical observations that in some patients with Von Willebrand disease (VWD), vascular malformations can cause severe gastrointestinal (GI) bleeding. In vitro and in vivo studies indicate that VWF can regulate angiogenesis through multiple pathways, both intracellular and extracellular, although their relative importance is still unclear. Investigation of these pathways has been greatly facilitated by the ability to isolate EC from progenitors circulating in the peripheral blood of normal controls and patients with VWD. In the next few years, these will yield further evidence on the molecular pathways controlled by VWF and shed light on this novel and fascinating area of vascular biology. In this article, we will review the evidence supporting a role for VWF in blood vessel formation, the link between VWF...
dysfunction and vascular malformations causing GI bleeding and how they may be causally related. Finally, we will discuss how these findings point to novel therapeutic approaches to bleeding refractory to VWF replacement therapy in VWD.

INTRODUCTION

1. Angiogenesis and haemostasis

Blood vessels control many biological processes that are essential for life. Two of these are angiogenesis, i.e. the formation of new blood vessels from pre-existing ones, and haemostasis, i.e. the ability to control bleeding by forming clots at sites of vascular injury. Historically, these two disciplines have been investigated separately, and only recently have the clear overlaps between the two been recognised. From a functional perspective, it makes sense that these two processes should co-operate physiologically, since they are often co-localised in time and space, such as during wound healing or embryonic development. Indeed in vivo studies show that mutation of genes essential for vascular development or haemostasis often result in both vascular defects and bleeding [1]. The ability to investigate the two processes through ever more sophisticated in vivo models has demonstrated that several haemostatic proteins such as tissue factor, prothrombin, Factor XIII and Factor V control the formation of blood vessels [2-8]. However in humans a major challenge in defining the role of haemostatic proteins in angiogenesis is the difficulty in establishing a specific readout, since vascular disruption and defective haemostasis both result in bleeding. Given the number of human diseases caused by defects in coagulation, understanding the possible roles of haemostatic proteins in blood vessel formation may have significant implications for the treatment of patients. This is particularly relevant in cases where replacement therapy, the traditional choice for coagulation disorders, is not effective in the management of bleeding.

2. Angiogenesis: basic concepts in health and disease

Angiogenesis is a complex, multistep process which involves numerous pathways acting in concert to produce a stable blood vessel (rev in [9]). Angiogenesis is essential for embryonic development. In the adult, the formation of new blood vessels is restricted to specific physiological processes such as the menstrual cycle, tissue repair and wound healing. Many regulators of angiogenesis both endogenous and pharmacological have been described and continue to be identified, suggesting that this process is very sensitive to modulation by intracellular and extracellular factors. To add to the complexity, the same factors or drugs may have opposite effects, namely anti- or pro-angiogenic, depending on the experimental model used [10, 11]. This may be partly related to the circular nature of the process, which post-development starts and ends with a stable blood vessel, through an intermediate phase of destabilization.

Of the many regulators of angiogenesis, some are essential for all or most blood vessels and therefore their deficiency is incompatible with life. Examples of these are growth factors of the
VEGF family [12] and transcriptional master regulators of endothelial lineage differentiation [13]. Many other proteins however, which modulate the process of new vessel formation, appear to be partly dispensable and possibly act in a tissue-restricted manner, so that their deficiency or defect results in malformations rather than complete absence of a vascular network. Examples of these are mutations in the WNT receptor FZD4 which cause familial exudative vitreoretinopathy [14], or coagulation factor XIII deficiency which can cause wound healing defects [15].

Numerous diseases are associated with increased angiogenesis: as well as tumors, many chronic inflammatory diseases, ocular diseases, diabetes and obesity involve enhanced, often abnormal angiogenesis[16]. Thus anti-angiogenic agents have shown various levels of clinical efficacy in a number of diseases. Conversely, the ability to promote neo-vascularization is the goal in diseases characterized by tissue ischemia, and is a major component of any tissue engineering approach. A less investigated area is that of vascular malformations, i.e. localized lesions of blood vessels that occur because of defects during angiogenesis. These range from trivial to life-threatening or disabling problems as a result of location, pain, tissue damage and bleeding [17]. Vascular malformations in the gastrointestinal tract, called angiodysplasia, are the most common cause of obscure gastrointestinal bleeding, which is the most frequent cause of anemia in adults [18]. The molecular mechanisms underlying the formation of angiodysplastic lesions are poorly understood, but a loss of balance between proliferation and stabilization during angiogenesis may result in excessive, unstable and dysfunctional new vessels [19].

3. Von Willebrand factor in haemostasis

VWF is a multifunctional glycoprotein best known for its essential roles in primary and secondary haemostasis, as a mediator of platelet adhesion and as carrier for coagulation Factor VIII. VWF is synthsised in endothelial cells (EC) and megakaryocytes and is present in three pools:

- Cellular VWF: present in vascular EC and in platelets, stored in Weibel Palade bodies and in alpha granules respectively and released upon stimulation.
- Plasma VWF: circulating as a large globular protein and derived almost entirely from EC release.
- Subendothelial VWF: arising from abluminal release from EC and bound to molecules of the extracellular matrix (ECM) and endothelial and vascular smooth muscle cell surface receptors.

The VWF monomer is 220 KDa, and contains multiple domains to which the many functions of VWF have been mapped [20, 21]. By a process of C- and then N- terminal linking, the monomers are assembled into large multimers of up to 20 000kDa. At the N terminus, the D'D3 domains bind to FVIII; the central A domains are involved in binding to platelet GP 1b (A1), heparin (A1) and collagen (A1 and 3). The A2 domain contains the cleavage site for ADAMTS13, which controls VWF’s multimeric size; the C4 domain contains the RGD sequence involved in binding to β3 integrins. Both the multimeric size and the conformation
of VWF determine its platelet binding activity. In plasma, VWF adopts a folded globular conformation that does not bind to its main platelet receptor GPIbα. Fluid shear stress or binding to surfaces such as collagen and the extracellular matrix exposes the buried binding site for GPIbα, localized in the VWF A1 domain. Unfolding of VWF also exposes the Tyr1605-Met1606 bond in the A2 domain facilitating its cleavage by the metalloproteinase ADAMTS13, which determines the size range of circulating VWF multimers [22]. Following vascular injury, VWF is released from the endothelium and can undergo a similar unraveling process while it remains tethered to the EC, mediating platelet adhesion to the endothelium and becoming susceptible to ADAMTS13 cleavage [23].


Congenital decrease or dysfunction of VWF causes Von Willebrand disease (VWD), the most common inherited bleeding disorder in humans (rev in [24, 25]). The classification of VWD, based on differences and degree of quantitative and qualitative defects has been thoroughly described. Deficiency of VWF function can also be acquired (acquired von Willebrand syndrome or AVWS), due to dysfunction or degradation of VWF, seen in association with clinical conditions such monoclonal gammopathy, myeloproliferative and malignant disorders, aortic valve stenosis and in patients with left ventricular assist devices (LVAD) (rev in[26]). In hereditary VWD, bleeding is generally controlled by replacement therapy with VWF-containing concentrates, but a number of reports have shown this to be less effective in gastrointestinal (GI) bleeding ([27, 28]), suggesting that additional mechanisms may be involved.

Up to 20% of patients with VWD present with gastrointestinal (GI) bleeding [29]; this can be severe and require repeated blood transfusions. Bleeding from this site seems to be particularly common when there is a deficiency of the larger VWF multimers, as in in type 2A VWD [27] and in AVWS [30]. GI bleeding has also been linked to the presence of angiodysplastic lesions in the GI tract and this has been reported in 2-4% of patients with VWD [29, 31]. As noted above, these are thought to develop due to dysregulated angiogenesis, leading to the production of fragile vessels prone to bleeding[19]. Bleeding from angiodysplasia has been reported to present as soon as 11 days following placement of a left ventricular device [32]. Interestingly, vascular malformations outside the GI tract have also been reported in patients with VWD [33, 34].

5. The multiple roles of von Willebrand Factor in the vasculature

Besides its well-characterised role in haemostasis, VWF has increasingly been implicated in other biological processes (rev in [35]). The formation of WPB in EC is absolutely dependent on the synthesis of VWF, but analysis of WPB contents has shown that they also contain a large number of vasoactive molecules. Many of these have been shown to bind to VWF, raising the possibility that VWF plays a role in directing and regulating their actions after
release with consequent effects on angiogenesis. In vivo studies have implicated VWF in the control of vascular inflammation and leukocyte recruitment [36], metastasis [37] and in the regulation of vascular permeability in the brain, with possible relevance for brain inflammatory pathologies and stroke [38-40]. The multiple functions of VWF have been reviewed elsewhere [35]. Thus as novel binding partners and novel functions of VWF are being identified, a complex network of interactions with multiple vascular pathways is emerging, indicating that VWF may be involved in the pathogenesis of vascular diseases well beyond the control of haemostasis.

6. Von Willebrand Factor regulates angiogenesis.

Our contribution to this field has been the identification of VWF as a regulator of angiogenesis [41], which has provided the first plausible explanation for the presence of vascular malformation in patients with decrease or dysfunction of VWF. Inhibition of VWF expression in human umbilical vein EC (HUVEC) using siRNA resulted in increased proliferation, migration and in vitro angiogenesis [41]. A similar overall pattern was found in blood outgrowth endothelial cells (BOEC) from patients with VWD although differences in the cellular phenotypes have been observed depending on the individual molecular defect [41, 42]. In vivo, angiogenesis and vascular density were found to be increased in the VWF deficient mouse, in several physiological and pathological models [41, 43]. Because of the numerous molecular interactions and functions of VWF in the vasculature, multiple molecular pathways are likely to be involved in the regulation of angiogenesis by VWF. So far, the evidence points to VWF modulating angiogenesis through both extracellular and intracellular pathways. A model of the molecular pathways through which VWF may regulate angiogenesis is shown in Figure 1.

6.1 VWF control of angiogenesis: extracellular pathways

In vitro, plasma-derived VWF inhibits endothelial tube formation in a basic model of angiogenesis [41], indicating the existence of an extracellular pathway. VWF binds to EC via integrin αvβ3 [44, 45], a heterodimeric adhesion receptor with multiple ligands, which plays a critical but complex role in angiogenesis and vascular homeostasis [46, 47]. Pharmacological inhibition of αvβ3 inhibits angiogenesis in experimental models; however, genetic β3 deficiency results in enhanced angiogenesis in vivo. Thus αvβ3 appears to exert a bimodal effect on angiogenesis, both as activator and inhibitor, playing different roles possibly depending on phases of angiogenesis, different ECM ligands, crosstalk and/or interaction with other receptors (rev in [48]).

Multiple pathways downstream of αvβ3 link this receptor to regulation of gene expression and crucially to vascular endothelial growth factor receptor-2 (VEGFR)-2 signaling. A complex, reciprocal relationship exists between VEGFR-2 and αvβ3 integrin in EC (rev in [49] and Figure 2). VEGFR2–αvβ3 integrin association is important for full VEGFR-2 activity and activation of downstream signaling [50]. However, lack of endothelial β3 causes over-
sensitivity to VEGF and increased VEGFR2 signaling leading to immature, fragile blood vessels [47, 51], similar to angiodysplastic lesions: indeed, a role for increased VEGF signaling has been proposed in angiodysplasia [52, 53]. VWF deficiency also results in enhanced VEGFR2-dependent endothelial migration and proliferation [41], suggesting that VWF normally controls angiogenesis by inhibiting VEGFR2 signaling. VWF also controls \( \alpha \nu \beta_3 \) surface expression by preventing its internalization [41]. The consequent net effect of VWF on the pathways above is unknown and requires further investigation.

Interestingly, the interaction of VWF with \( \alpha \nu \beta_3 \) may also affect another aspect of vascular development, namely arterial maturation. Using a mouse model of retina vascular development, Scheppke et al reported that arterial maturation was delayed in VWF-deficient mice. In an elegant study, they showed that Notch signaling from EC to vascular smooth muscle cells (VSMC) leads to expression of integrin \( \alpha \nu \beta_3 \) in VSMC. This would then interact with sub-endothelial VWF leading to vessel maturation [54]. These data add to the evidence that VWF is required for physiological angiogenesis, possibly acting at multiple stages of blood vessel development.

The observation that vascular malformations are most frequent in patients with AVWS and with type 2A VWD suggests that VWF high molecular weight multimers (HMW), which are crucial for haemostasis, may also be critical for the control of angiogenesis. The reasons for this are presently unknown. It is possible that HMW multimers may enhance the interaction between VWF and its endothelial receptors, thus potentiating the signaling effect—for example by clustering of \( \alpha \nu \beta_3 \) and/or mediating crosslinking to multiple cell surface receptors. Moreover, VWF can interact with circulating molecules which affect angiogenesis; it is possible that the larger multimers may enhance the affinity for these molecules to their receptors. [55] It is also conceivable that platelets, recruited by HMW VWF multimers, may contribute angiogenesis regulators which are essential for physiological blood vessel growth and stabilization.

6.2 VWF control of angiogenesis: intracellular pathways

VWF drives the formation of WPB, the endothelial storage organelles which contain multiple proteins, including the angiogenesis regulator Angiopoietin-2 (Ang-2) [56, 57]. Ang-2 is part of the Angiopoietins/Tie-2 pathway, a crucial system regulating vascular homeostasis and angiogenesis [58]. Ang-2 can act to destabilize blood vessels and synergise with VEGF to promote angiogenesis [59]. In vitro studies show that VWF regulates the endothelial storage and release of Ang-2: inhibition of VWF expression results in loss of WPB with increased release of Ang-2 in the cell supernatant [41]. Interestingly, this is not a general effect on all WPB proteins, as IL-8 release is not increased in VWF-deficient cells (Starke and Randi, unpublished). VWF also controls Ang-2 synthesis: loss of VWF in vitro in HUVEC (Smith and Randi, unpublished) and in heart tissue from VWF-deficient mice [60] results in enhanced Ang-2 mRNA levels. Moreover, BOEC from a patient with type 3 VWD and
complete lack of endothelial VWF synthesis also show enhanced Ang-2 synthesis and release [61]. Whether Ang-2 plays a role in the enhanced, disrupted angiogenesis caused by lack of VWF in experimental models, and crucially in the pathogenesis of angiodysplasia in VWD patients, remains to be determined. Recently, an association between Ang-2 and sporadic angiodysplasia has been identified, with raised Ang-2 levels in plasma and tissues [62].

6.3 VWF-dependent platelet adhesion: potential role in angiogenesis

VWF has a well-established role in mediating platelet capture at sites of vascular damage and inflammation, especially under conditions of high shear stress. The platelets may then be bound onto the surface of endothelial cells or to the subendothelial matrix. The process of capture and subsequent binding interactions results in platelet activation with release of numerous vasoactive mediators. These are thought to be important in vessel wall repair and healing and also to be one of the first steps in atherogenesis. A reduction in atherosclerosis was noted in the VWF-null mouse [63], but it is not clear that any such protective effect occurs in humans [64]. It is also possible that failure of this mechanism in VWD may contribute to angiodysplasia. Angiodysplastic lesions are not a feature of the major platelet function defects or of thrombocytopenia [65]; however angiodysplasia has been reported in patients with Glanzmann thrombasthenia [66] and Bernard-Soulier syndrome [65]. Detailed reviews of the clinical data possibly associated with vascular malformations in platelet dysfunction would be important.

7. Treatment of angiodysplasia in VWD

Intestinal blood loss from angiodysplasia in patients with VWD has been frequently recognized as a difficult and sometimes intractable problem. This is in part due to the often widespread and recurrent nature of the angiodysplastic lesions which makes local or surgical approaches of limited efficacy but also due to the nature of the lesions. The relative excess of GI bleeding in type 2A compared to 2M points to an importance of HMW in haemostasis at this site and clearly it is difficult to sustain high levels of HMW using intermittent infusions due to the persistent action of ADAMTS13. Recombinant VWF, which has not been previously exposed to ADAMTS13 contains ultra-large multimers, but these are also reduced by cleavage within a few minutes of infusion [67]. Not surprisingly therefore, published reports indicate that replacement therapy for GI bleeding is less effective than for bleeding at other sites and that higher doses of concentrate are required [27]. It is also possible that the lack of platelet, EC and subendothelial VWF is particularly important for hemostasis in the GI tract. Thus a significant proportion of patients remain dependent on frequent red cell transfusions despite conventional local and systemic treatments. Moreover, for those patients with AVWS, replacement therapy is inevitably ineffective and so attention has focused on ways to induce regression of the angiodysplastic lesions themselves.
Early reports of success in treating angiodysplasia with oestrogens and progesterones were not reproduced in controlled trials [19, 68]. However, Tamoxifen (an anti-oestrogen) was shown in a double-blind randomised and placebo-controlled trial to reduce epistaxis in patients with HHT [69]. It has subsequently been reported to also benefit patients with VWD and angiodysplasia [70]. Thalidomide is an agent with established anti-angiogenic properties, interestingly mediated by suppression of VEGF, and in a randomised trial yielded a response rate of 71% in patients with refractory bleeding from vascular malformations compared to a rate of 4% from iron therapy. Response was associated with a reduction in plasma VEGF [71]. Responses have also been reported in VWD-associated angiodysplastic bleeding, but its use is frequently limited by the development of neuropathy. Most recently, success has been reported with atorvastatin which is also known to have anti-angiogenic effects at higher doses [72, 73]. The reduction in bleeding has been significant, but regression of angiodysplasia has not been directly demonstrated and it has not been subjected to a randomised trial. A number of anti-VEGF, strategies have been developed, including anti-VEGF and anti-VEGFR antibodies, VEGF-trap and small molecule inhibitors (angiostatin, endostatin). These agents were developed for use in patients with malignancy, but GI and nasal bleeding was a frequent complication, indicating that they may not be useful in patients with haemostatic defects.

The development of models of angiodysplasia would undoubtedly help the development of appropriate treatments. However, no specific in vivo model is described in the literature. In the absence of relevant models on the GI system, since vascular malformations in VWD patients are not uniquely localised in the gut but are also present in the nail bed, it may be possible to develop a mouse model where analysis of vascular malformations in the skin can be used as surrogate. It is difficult to envisage an in vitro model that could capture the complexity, including the mechanical stress, of the environment where these lesions are found. However, cellular models of angiogenesis could be modified to make them more sensitive to growth factor imbalance, as described in tissue samples from patients.

Conclusions and future directions
The identification of a role for VWF in the control of blood vessel formation adds to the increasing web of roles and functional interactions that this large and fascinating protein can engage in. Although clinical evidence from nearly a century and innumerable studies in patients and models clearly show that the key role of plasma VWF is to control haemostasis, other functions, including the modulation of angiogenesis, may provide new prospects for the understanding and ultimately the care of patients with VWD. In the near future, the generation of animal models to investigate the molecular links between lack or dysfunction of VWF and angiodysplasia should contribute very valuable tools in the development of novel candidate targets for the treatment of this unresolved clinical issue.
Figure legends

**Figure 1: von Willebrand factor (VWF) controls angiogenesis and vascular maturation through multiple pathways: model**

*Left panel:* within endothelial cells (EC), VWF regulates endothelial proliferation, migration and angiogenesis through pathways which converge to control vascular endothelial growth factor receptor (VEGFR)-2 signalling. VWF is essential for the formation of WPB, organelles which store the growth factor Angiopoietin (Ang)-2. VWF controls Ang-2 levels by promoting its storage and inhibiting its synthesis. Ang-2 is released upon EC activation and synergizes with VEGFR-2 signaling to destabilize blood vessels and promote angiogenesis. Moreover, VWF can interact with integrin αvβ3, a heterodimeric adhesion receptor with multiple ligands, involved in angiogenesis and vascular homeostasis. VWF binding to αvβ3 integrin stabilizes its expression on the EC surface. In EC, αvβ3 integrin can repress VEGFR-2 activity and downstream signaling. *Right panel:* during vascular development, expression of αvβ3 can be upregulated in vascular smooth muscle cells (VSMC). VWF binding to αvβ3 on VSMC is required for their recruitment, thus promoting arterial maturation during vascular development. Therefore, loss of VWF results in disrupted blood vessel formation through multiple pathways, involving both EC and VSMC.

**Figure 2: Functional cross-talk between VEGF Receptor-2 (VEGFR2) and integrin αvβ3 on the endothelium.**

The model shows a simplified schematic of the complex cross-talk and functional interaction between VEGFR2 and αvβ3. As discussed in the text, VEGFR2–αvβ3 integrin association is important for full VEGFR-2 activity and activation of downstream signaling. Activation of VEGFR2 by VEGF results in VEGFR2 phosphorylation and recruitment of adaptors including c-src, which phosphorylate the cytoplasmic domain of αvβ3 and promote integrin activation. This in turn results in conformational changes and increase in ligand binding affinity, which triggers outside-in signalling and further activation. However, lack of endothelial β3 causes over-sensitivity to VEGF and increased VEGFR2 signaling, suggesting that αvβ3 is also capable of inhibit VEGFR2 activity, possibly based on its ligand occupancy. This might be one of the effects of VWF on this signalling pathways. A detailed review on this topic can be found in Ref [49].
References


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