Pathophysiology of von Willebrand factor in bleeding and thrombosis

Pagliari, M.T.

CHAPTER 1

INTRODUCTION AND OUTLINE OF THE THESIS

VON WILLEBRAND FACTOR SYNTHESIS

von Willebrand factor (VWF) is a multimeric glycoprotein principally synthesized in the endothelial cells and in a lower percentage in megakaryocytes [11]. VWF is synthesized as a pre-pro-polypeptide of 2813 amino acids (aa) that includes a signal peptide (22 aa), a propeptide (741 aa), and the VWF (2050 aa), which undergoes several post-translational modifications before it reaches its mature form [2-5]. The first series of modifications occur in the endoplasmic reticulum (ER) where the signal peptide is cleaved and VWF is glycosylated at N-linked glycosylation sites. Then, the subunits dimerize through the formation of disulfide bonds at the carboxyl-terminal extremity [6, 7]. Once in the Golgi, further glycosylation by adding O-linked glycans occurs along with sulfation.

At this stage, the VWF propeptide (VWFpp) is cleaved by furin. The VWFpp remains non-covalently bounded to VWF exerting an important regulatory role in driving the multimerization process which occurs through the formation of disulfide bonds involving the N-terminal extremity of dimers [6, 8].

The VWFpp also regulates the storage of mature VWF within the Weibel Palade Bodies (WPBs) in endothelial cells or the package into alpha-granules in platelets [9]. The mature VWF can be secreted in absence of stimuli (basal secretion [9-11]) or it can be released after endothelial or platelet activation (regulate secretion [12]).

VON WILLEBRAND DISEASE

von Willebrand disease is an inherited bleeding disorder caused by the presence of a mutation in *VWF* [13] and it can be distinguished in quantitative or qualitative defects. Quantitative defects include a partial deficiency of VWF defined as type 1 VWD, whereas the virtual absence of VWF is referred to as type 3 VWD. These two forms differ for the severity of the quantitative reduction and for the inheritance pattern which is autosomal dominant in the former and recessive for the latter.

The qualitative defects, mainly inherited with a dominant pattern, are generally recognized as type 2 VWD and can be further divided into four subgroups. Briefly, type 2A includes those variants characterized by an altered multimeric pattern with lacking of the most hemostatically active high molecular weights multimers (MWM) and in most severe forms even intermediate-MWM are missing. Type 2B includes those variants with increased VWF capacity to interact with glycoprotein Ib (GPIb) complex exposed on platelets surface, thus resulting in a faster consumption of MWM. Type 2M includes those variants responsible for an altered VWF function with reduced platelet or collagen binding activity although in presence of a normal multimeric pattern. Lastly, type 2N is caused by variants affecting VWF capacity to bind Factor (F) VIII [13, 14].

AVAILABLE TREATMENTS FOR VWD

Bleeding manifestations are due to either quantitative or qualitative VWF defects and in a second stage to the reduced FVIII levels. Therefore, the therapeutical approach focuses

on the normalization of VWF activity levels [15, 16]. The most common treatment consists of the infusion of the desmopressin (1-deamino-8-d-arginine vasopressin, DDAVP) which stimulate the transient release of both VWF and FVIII [17, 18]. The therapeutic effect of DDAVP should be tested by a trial administration to evaluate patient-individual responsiveness. Generally, it is administrated to type 1 patients with exception e.g., for those with an increased VWF clearance or when a prolonged treatment is required. Even a few type 2 patients may benefit from this treatment, such as in case of minor bleeding [19], although it is contraindicated in 2B as the rise in circulating mutant VWF with increased capacity to bind platelets may cause thrombocytopenia. Self-evident, DDAVP is not effective in type 3 patients because of the complete lack of VWF [20].

For these exceptions and in case of major and prolonged bleeding, regardless of VWD classification, the use of concentrate containing VWF is recommended. Depending on their origin, we can distinguish between plasma-derived (pd) VWF and recombinant (r) VWF. Pd-VWF can differ by the source, the purification process, the method used for viral inactivation and the variable amount of FVIII in the concentrate. The rVWF is produced in Chinese hamster ovary cells. Due to the method of production and the lack of ADAMTS13 cleavage activity, the rVWF shows ultra-large (UL) VWF multimers with a longer half-life than pd VWF products and it does not contain FVIII [21-23].

In some cases, patients may be treated with other additional drugs like tranexamic acid, a fibrinolysis inhibitor that can be administrated either alone or in combination with both DDAVP and VWF concentrate to treat minor mucocutaneous bleeding or in case of dental surgeries [24-26].

VWF BETWEEN BLEEDING AND THROMBOSIS

The most known function of VWF consists in the maintenance of the primary hemostasis exerted through the recruitment of platelets and by mediating their adhesion at the site of vascular damage [1, 27]. Indeed, the VWF can recognize and bind the different types of collagen exposed after the damage through the A3 (collagens I and III [28, 29]) and A1 (collagen IV and VI) domains [30, 31]. After anchoring to the vessel, the VWF is stretched by the shear which allows the exposure of A1 domains and as a consequence their interaction with the GPIb complex on platelets. Nevertheless, the VWF also plays a role in the secondary hemostasis circulating in a complex with FVIII stabilizing and protecting it from the proteolytic degradation [1].

The multimeric structure of VWF is strongly correlated with its hemostatic activity, in which the UL multimers can spontaneously bind to platelets [32]. The regulation of the pro-hemostatic function of VWF and thereby the prevention of spontaneous thrombosis is exerted by the disintegrin and metalloprotease with thrombospondin Type 1 repeats 13 (ADAMTS13) that is primarily synthesized in the hepatic stellate cells [33]. In physiological conditions, ADAMTS13 binds to the VWF A2 domain cleaving the Y1605-M1606 bond and resulting in smaller and less functional VWF molecules [34].

Severe deficiency of ADAMTS13 may cause thrombotic thrombocytopenic purpura (TTP), a life-threatening disorder characterized by the formation of thrombi rich in platelets and VWF [35]. On the contrary, the presence of mutations localized within the A2 domain may be responsible for an increased susceptibility of VWF to proteolysis by ADAMTS13 as described in patients with type 2A VWD with loss of VWF high- MWM [36].

The perturbation of the equilibrium between VWF and ADAMTS13 plasma levels has been investigated in the onset of different thrombotic disorders. The increase of plasma VWF and FVIII levels have been independently reported as associated with the risk of venous thromboembolism (VTE) [37-40]. Similarly, a reduction of ADAMTS13 levels has also been investigated in the onset of different thrombotic disorders including myocardial infarction, coronary artery disease, and venous thromboembolisms [41-43]. Nevertheless, the complete mechanism which links the increase of VWF and FVIII levels and a decrease of ADAMTS13 still remains to be clarified.

OUTLINE OF THE THESIS

This thesis focuses on the role of VWF in bleeding and thrombosis. In the first part of the thesis, the role of VWF variants and the development of VWD are considered. The second part focuses on type 3 VWD, the most severe form of this disease caused by the complete lack of VWF. Finally, the third part considers the VWF as a risk factor in the onset of deep vein thrombosis either alone or in combination with changes in ADAMTS13.

Part I: Genetics of von Willebrand disease

Chapter 2 describes the approach used to perform a differential diagnosis between type 2A and 2B VWD. For this purpose, the platelet-dependent VWF activity (VWF:GPIbM) over the VWF ristocetin cofactor activity (VWF:RCo) ratio has been used as an alternative to the ristocetin-induced platelet aggregation (RIPA).

In **Chapter 3**, a group of five patients carrying the type 1 variant p.Arg1379Cys has been characterized. One was diagnosed as affected by type 1 VWD, whereas the four who also carried the polymorphism p.Ala1377Val *in cis* with p.Arg1379Cys had a type 2M phenotype. The *in vitro* expression study evaluating the capacity of the wild-type, mutants, and hybrid recombinant (r) VWF to bind recombinant GPIb in presence of an increasing concentration of ristocetin showed that their synergistic effect is the cause of type 2M phenotype.

The genotypic characterization of two unrelated Italian VWD patients has been described in **Chapter 4**. The *novel* variant p.Thr274Pro found at heterozygous state has been expressed *in vitro* and rVWF localization has been further evaluated using immunofluorescence. This contributes to explain that the reduction of VWF levels measured in patients' plasma was caused by a combination of reduced synthesis, impaired secretion and multimerization.

Part II: Type 3 von Willebrand disease: results from the 3WINTERS-IPS

Chapter 5 focuses on the role of VWFpp in the determination of the pathomechanism underlying type 3 VWD in the largest cohort of type 3 VWD patients so far collected, the 3WINTERS-IPS. In this study, we showed that the VWFpp over VWF antigen (VWF:Ag) ratio may discriminate between homozygous/compound heterozygous carriers for a *VWF* missense variant and homozygous/compound heterozygous carriers of *VWF* null alleles. The use of FVIII over VWF:Ag ratio, however, failed to discriminate missense from null defects.

In **Chapter 6**, the development of alloantibodies against VWF in the 3WINTERS-IPS cohort has been investigated. We confirmed that the development of neutralizing antibodies represents a rare side effect of the replacement therapy with VWF concentrates and it is mainly found in type 3 patients homozygous for null defects. Because VWF inhibitors may recognize different epitopes on VWF, the prevalence estimation is strongly affected by the type of assay used.

Part III: Role of von Willebrand Factor in deep vein thrombosis

In the last two chapters, we have investigated the role of ADAMTS13, VWF, and FVIII as risk factors for deep vein thrombosis (DVT). In **Chapter 7** we demonstrated that a slight decrease in ADAMTS13 activity levels or an individually increase in VWF and FVIII levels are associated with an increased DVT risk. Moreover, we showed how the combination of slightly reduced plasma ADAMTS13 activity levels combined with high VWF levels is responsible for a marked increase in DVT risk in our study population. Subsequently, in **Chapter 8** we investigated the genetic component of DVT, focusing on *ADAMTS13*, *VWF* and *F8*. In this work, we applied a targeted next-generation sequencing approach to evaluate the association of common, low-frequency, and rare variants located in the coding region of these 3 genes with the onset of DVT. Finally, **Chapter 9** will include a general discussion and conclusions about the topics described in this thesis focusing on the future perspectives including a new cellular model to study and characterize VWF.

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