Chapter 9

Summary

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总结

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Biogenesis of the endothelial cell-specific organelles Weibel-Palade bodies (WPB) is driven by von Willebrand factor (VWF) [1]. WPB serve as an intracellular pool for many bioactive molecules including P-selectin and tissue plasminogen activator (t-PA) and the hemostatic protein VWF [2]. Mutations in VWF may impair WPB biogenesis and exocytosis and lead to the most common inherited bleeding disorder in humans, von Willebrand disease (VWD) [3].

Even though the biogenesis and biological significance of WPB has been studied extensively in the past, the pathophysiology of VWD remained poorly understood. The pathogenic effects of VWF mutations have been studied in detail, but these studies were limited to analysis of constitutive VWF secretion and multimerization. This was probably due to the lack of suitable cell model-systems to study the effects of VWF mutations on WPB formation and exocytosis. In **Chapter 2**, we have reviewed the role of VWF in the formation of WPB and discussed the studies of VWF mutations in the context of VWD. Defects in VWF secretion lead to a quantitative deficiency of VWF and contribute to the bleeding tendency as seen in VWD [4]. The pathophysiology of VWF mutations has been extensively evaluated in several heterologous cell lines such as COS cells in which VWF is not stored. Since *in vivo* VWF is mainly stored in WPB and secreted from vascular endothelial cells, cell lines like COS cells can not recapitulate all aspects of VWF biology. Human umbilical vein endothelial cells (HUVECs) are better suited and have been obtained to study VWD [5-9], but these cells can not be widely used because the access to HUVECs with specific VWF mutations is very limited at best. Emerging evidence shows that HEK293 cells, in which VWF is stored in WPB-like organelles [10], and patient-derived peripheral blood endothelial cells (BOECs) are more suitable tools for such studies (**Chapters 3 and 6**).

In **Chapter 3**, we characterized the morphology and function of pseudo-WPB formed in HEK293 cells upon expression of VWF and showed that these pseudo-WPB resemble endothelial WPB in many aspects. By transient expression of VWF mutations associated with quantitative VWF deficiency in HEK293 cells, we demonstrate that four missense mutations in the D3 and CK-domain of VWF diminished the storage in pseudo-WPBs, and led to retention of VWF within the endoplasmic reticulum (ER). VWF variant p.Cys1060Tyr formed slightly fewer pseudo-WPB compared to WT-VWF, whereas p.Cys1149Arg, p.Cys2739Tyr and p.Cys2754Trp formed few and abnormal pseudo-WPB. Consequently, these three mutations led to severe defects in regulated secretion of VWF. These results

validated the feasibility of HEK293 cells for the study of the pathogenic nature of VWF mutations with respect to intracellular storage and regulated secretion and confirmed the pivotal role of the VWF D3 domain in the formation of WPB [11,12]. In addition, this study suggests that mutations in the CK domains and not only those in the D'D3 domains affect WPB biogenesis.

Some type 1 VWD patients are known to have a reduced response to DDAVP treatment [13]. Interestingly, most of the VWF mutations identified in those patients were located within the A1-A3 domains. As the DDAVP-induced increase in plasma VWF is presumably due to exocytosis of WPB [14], the formation of WPB in those patients might be impaired by VWF mutations. To confirm this hypothesis, we analyzed the effects of quantitative VWF mutations in the A domains on the intracellular storage and regulated secretion of VWF in **Chapter 4**. We showed that five of the six mutations indeed impaired pseudo-WPB formation, and that in particular VWF mutations p.Leu1307Pro and p.Val1822Gly reduced the regulated secretion of VWF, both in homozygous and heterozygous state. Both p.Leu1307Pro and p.Val1822Gly were identified in patients with a reduced response to DDAVP, indicating their deleterious effects on the biogenesis of WPB *in vivo*. The decrease in regulated secretion of VWF caused by p.Ser1285Pro predicts a poor response of the patients to DDAVP, but no data are available yet. Furthermore, p.Arg1374His, p.Arg1583Trp and p.Tyr1584Cys showed normal secretion of VWF although p.Arg1374His and p.Tyr1584Cys disrupted the morphology of pseudo-WPB. The pathogenic role of p.Arg1374His is not conclusive in this study. The effects of the mutations p.Ser1285Pro, p.Leu1307Pro and p.Tyr1584Cys on WPB formation and regulated secretion as seen in transfection experiments with HEK293 cells were confirmed in the endothelial cells (BOECs) derived from the corresponding patients in **Chapter 6**.

Cysteine mutations in VWF have been frequently identified in VWD patients (mutation database www.vwf.group.shef.ac.uk/). While most of these cysteine mutations were identified in type 1 or type 3 VWD, only five were identified in type 2 VWD. Intriguingly, all of these five mutations lead to the loss of one of the three specific cysteines - p.Cys1099, p.Cys2771 and p.Cys2773 - that are involved in the interchain disulfide bonds of VWF [15,16]. All the other cysteine mutations probably disrupt the intrachain disulfide bonds of VWF [15,16] and are associated with reduced plasma VWF levels (**Chapter 5**). These distinct effects of the two types of cyteine mutations made us hypothesize that impaired intrachain or interchain disulfide bond formation in VWF may have different effects on the biogenesis of

WPB. By analyzing formation of pseudo-WPB upon expression of VWF variants with disrupted intrachain (p.Cys1130Phe and p.Cys2671Tyr) or interchain (p.Cys2773Ser) disufide bond formation in Chapter 5, we concluded that natural mutations of cysteines involved in the formation of interchain disulfide bonds do not affect the storage in WPB and secretion of VWF, whereas mutations of cysteines forming intrachain disulfide bonds will lead to reduced VWF storage and secretion due to ER retention.

Even though HEK293 cells are a promising cell model to study the structure-function relation of VWF, some limitations remain. Overexpression of VWF protein and difficulties with mimicking the heterozygous state make it difficult to address some questions in this system. A more ideal model system would be based on endothelial cells derived from the patients themselves. As the access to patients' HUVECs is very limited, we explored the feasibility of using BOECs - the endothelial cells derived from patients' peripheral blood - for the study of the pathogenic nature of specific VWF mutations (Chapter 6). We established that it is indeed feasible to obtain BOECs from multiple VWD patients and to recapitulate the phenotype of the patient in this cell model. We showed that BOECs provide a good model-system for the study of VWF string formation and for testing the response of endothelial cells to different stimuli. One interesting finding was that BOECs do not respond to DDAVP stimulation. A main limitation of this model-system remains the difficulty in accurately assessing the basal secretion of VWF because of variations between clones and cell passages. In addition, studies rely on the availability of patients and on the zygosity of VWF mutations in the patients. In these aspects, HEK293 cells are much flexible.

As demonstrated in Chapters 2-5, HEK293 cells are useful for the study of the intracellular storage in pseudo-WPB and the regulated secretion of VWF. In Chapter 7 we explored the possibility to study VWF string formation in this cell model. Even though all previous studies suggested that VWF strings were specific for endothelial cell, we for the first time showed that VWF strings can also be formed on HEK293 cells. These strings bind platelets under flow like the endothelial VWF strings do. By virtue of this novel system, we demonstrated that neither P-selectin nor alphaVbeta3 integrin are required for VWF string formation. Furthermore, structural changes in VWF were shown to modulate VWF string formation.

VWF strings are composed of ultra-large VWF multimers and are formed on activated endothelial cell surfaces after exocytosis of WPB. VWF strings recruit

platelets, thereby promoting clot formation and tissue repair [17]. Complete or partial loss of VWF string formation may contribute to the bleeding tendency of VWD. We therefore in Chapter 8 studied the effects of VWF mutation p.Arg1205His in two VWD type Vicenza patients: a female patient heterozygous for p.Arg1205His has a very mild phenotype and a male patient compound heterozygous for p.Arg1205His and p.Arg924Gln shows a severe phenotype. We demonstrated, in both HEK293 and BOECs model-systems, that p.Arg1205His reduced VWF production and secretion whereas p.Arg924Gln was deleterious for VWF string formation. These results indicated that the p.Arg1205His related low plasma level of VWF is not only caused by fast clearance of VWF but also by low intracellular production of VWF. The impairment in VWF string formation by p.Arg924Gln may contribute to the more severe phenotype in the patient who is compound heterozygous for p.Arg1205His and p.Arg924Gln. Why VWF mutation p.Arg924Gln reduced string formation needs further study. Although p.Arg924Gln slightly reduced the regulated secretion of VWF, it did not show apparent defects in the formation of pseudo-WPB, we therefore postulate that this mutation may affect string anchorage on the cell surface.

Conclusions and perspectives

The studies presented in this thesis explore several pathogenic mechanisms underlying VWD that is characterized by a quantitative VWF deficiency, in particular with respect to intracellular storage in WPB and regulated secretion of VWF. These studies advanced our understanding of VWD at the molecular and cellular level. HEK293 cells and BOECs were established as two useful model-systems for examining VWF structure-function relationships in the context of VWD. Using these model-systems we have demonstrated that VWF mutations may impair VWF storage and secretion and thus lead to a quantitative deficiency of VWF in the patients (Figure 1). Such a mechanism has also been confirmed by other groups [10,18,19]. Furthermore, we demonstrated that alteration in the structure of VWF, by natural mutations that occur in VWD patients, modulates VWF string formation and function. Under normal shear stress (10~70 dynes/cm^2 in arteries and 1~6 dynes/cm^2 in veins [20]), soluble VWF in plasma rarely binds platelets, whereas VWF strings formed on the activated endothelial surface are able to spontaneously recruit platelets from flowing blood thereby initiating clot formation at sites of vascular injury. Hence, alteration in VWF string formation and function may contribute to the bleeding tendency in VWD.

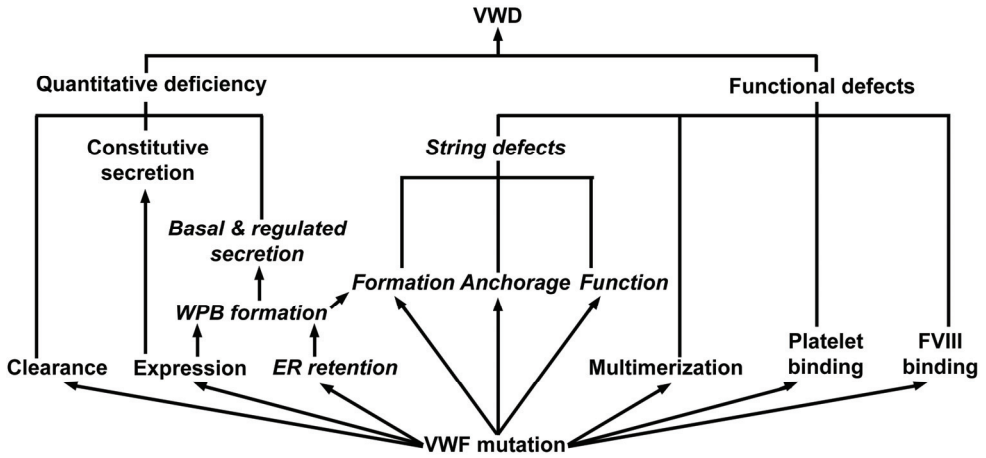

Figure 1. Proposed pathophysiological mechanisms underlying VWD. VWF mutations lead to quantitative or functional defects in VWF via complex mechanisms, and consequently to VWD. This thesis provided new mechanisms (indicated by italic font) underlying this bleeding disorder: defects in WPB formation and exocytosis, and defects in VWF strings (formation, anchorage and function). Of note, constitutive secretion pathway refers to secretion of VWF directly from the ER; basal secretion pathway refers to secretion of VWF from WPB under steady conditions (no stimulation); regulated secretion pathway refers to secretion of VWF from WPB under certain stimulation. VWF, von Willebrand factor; VWD, von Willebrand disease; and WPB, Weibel-Palade body.

VWF released from endothelial WPB via basal secretion is presumably the main source of soluble plasma VWF [21,22]. VWF strings formed upon (induced) exocytosis of WPB attach to the endothelial cell surface and recruit platelets thereby mediating platelet adhesion and aggregation. Defects in either of these two processes may contribute to the phenotype of VWD. Therefore, for the future studies of VWD, we propose that: 1) the pathophysiological effects of VWF mutations should be analyzed in cell models such as HEK293 cells and patient-derived BOECs in which VWF is stored in (pseudo-)WPB as *in vivo*; 2) in addition to multimer analysis, WPB formation and exocytosis, and formation and function of VWF strings should be included to fully document the pathogenic effects of VWF mutations; 3) as WPB also serve an intracellular pool for several inflammatory mediators such as P-selectin and angiopoietin-2, and as VWF *per se* may also regulate angiogenesis [23], a possible alteration in inflammation and vascular remodeling in VWD patients might be analyzed as well. We propose that inclusion of these three aspects in the analysis of VWF mutations will help physicians to

better understand the complex mechanisms underlying the phenotype of VWD patients, which will benefit the patients in the future.

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