Summary & Outlook

Summary

Endothelial cells line the interior surface of blood vessels and form the barrier between the blood and the underlying tissue. They are involved in variety of processes that include the regulation of blood tone, inflammation, angiogenesis and controlling hemostasis.[1] In many of these processes the endothelium rapidly secretes bioactive molecules from its storage organelles, the Weibel-Palade Bodies (WPBs).[2,3] The main component of the WPB is Von Willebrand factor (VWF).[4] This adhesive protein is involved the first steps of hemostasis and recruits platelets to sites of vascular damage to arrest bleeding. Mutations in VWF can lead to impaired hemostasis and cause the most common hereditary bleeding disorder Von Willebrand disease (VWD).[5] To understand the biological processes underlying VWD it is vital to know how VWF is produced, stored and secreted in healthy endothelial cells. In the WPB, the VWF is stored in characteristic tubules which give the WPB a distinct elongated shape.[2,6,7] The capability of VWF to fold in such a tubular conformation for storage is thought to be a hallmark for good VWF functioning.[5] In this thesis advanced electron microscopy techniques were used to study the morphological characteristics of VWF during storage and secretion with the aim to obtain novel insights on the molecular mechanisms that may cause in VWD.

One of the major imaging techniques that we performed to study VWF is Correlative Light and Electron Microscopy (CLEM). With CLEM fluorescent labeling is used to locate structures of interest for structural characterization by electron microscopy which was in our case Transmission Electron Microscopy (TEM). **Chapter 2** describes in detail our approach to combine fluorescent light imaging with TEM to relocate and identify the morphology of fluorescently labeled structures related to VWF secretion. To perform CLEM, we employed specially designed cell culture dishes that contain a glass bottom with an engraved coordinate system which functioned as a tool to accurately determine the location of the cell of interest with light microscopy. Subsequently, this coordinate system

is used during the TEM sample preparation protocol to obtain thin sections of exactly the same area to enable TEM analysis. We show that this technique can be combined with electron tomography to create a three dimensional reconstruction of the exocytosis event. Adapted versions of this protocol were used to study WPB biogenesis (**Chapter 4**) and VWF co-storage with Factor VIII (**Chapter 5**).

To visualize the structural characteristics of the mechanisms that act during WPB biogenesis or VWF secretion in three dimensions we explored Serial Block Face-SEM (SBF-SEM), a relatively novel electron microscopy technique. In **Chapter 3** we show the great potential of this technique but also point out some of the current limitations that need to be challenged to make optimal use the technique in WPB research.

In **Chapter 4** we investigated WPB biogenesis by performing immuno-gold labeling on TEM sections, by CLEM and by SBF-SEM. For CLEM we used transfected cells in which the WPBs are tagged with GFP enabling us to follow and investigate WPB biogenesis as a function of time. We show that WPBs are completely formed at the Golgi apparatus. Our data provides evidence that WPB formation is initiated in a Golgi stack where concentrated VWF is remodeled into tubules. During formation, the WPB emerges partially from the Golgi but stays connected via numerous membranous connections. In addition we observed dense clusters of material close to these connections. We argue that these clusters represent non-tubular VWF which will be incorporated in the WPB, facilitating growth.

In **Chapter 5** we investigate the structural organization of VWF in WPBs of endothelial cells that also express Factor VIII. In these cells Factor VIII is stored together with VWF in WPBs. This co-storage of VWF and Factor VIII changed WPB morphology and resulted in rounded vesicles. We show that these spherical WPBs contain short and disorganized VWF tubules which suggests that Factor VIII interferes in VWF tubule formation. In addition we show that the VWF from the Factor VIII containing WPBs still forms VWF strings upon stimulation. However, these strings show a reduced capability to bind platelets. This reduced binding seems to be caused either by bound Factor VIII that blocks the platelet binding site or by a Factor VIII induced conformational change in VWF that reduces the binding of platelet.

Adequate formation of functional VWF strings is essential for the first steps in primary hemostasis. In order to form functional VWF strings sufficient secretion of VWF is required. In **Chapter 6** we describe a relatively novel secretion

mode that allows endothelial cells to release large quantities of VWF. In stimulated endothelial cells we observed large VWF containing vesicles (1-2 micron in diameter) that were in close vicinity with WPBs and appeared to be the source of VWF strings. We show that these vesicles are formed prior to secretion which indicated multigranular exocytosis of WPBs. During multigranular exocytosis, secretory vesicles fuse first with each other before fusing with the plasma membrane. Therefore, we termed these large VWF vesicles "secretory pods". Using fluorescent VWF antibodies we could fix VWF to the site of exocytosis and revealed subsequently by CLEM that secretory pods are indeed the underlying structure from which VWF is released.

In **Chapter 7** we show by live-cell imaging the process of WPB fusion and the formation of secretory pods. We also investigated how this VWF is eventually remodeled into VWF strings upon secretion from secretory pods. With a second set of CLEM experiments, in which VWF string formation was not hampered by antibodies, we reveal that secreted VWF initially adopts a globular shape upon exocytosis. We also show that VWF is subsequently remodeled into strings by the shear stress induced by fluid flow and upon sufficient VWF anchoring to the cell surface. The observation that VWF is initially secreted as a dense globular mass and that its function relies on anchoring and unfolding provided novel pathophysiological mechanisms that could be affected in VWD.

Outlook

This thesis presents novel insights regarding WPB formation and VWF secretion, including the formation of VWF strings. In addition, we provide evidence for new pathophysiological mechanisms that may be effected in VWD. Our findings were obtained by exploring WPBs in healthy endothelial cells using various microscopy techniques.

In this thesis we also demonstrate the power of advanced imaging techniques such as CLEM. CLEM often played a central role in our research as it is a powerful tool based on fluorescent labeling and high resolution electron microscopic imaging to reveal specific morphological stages during WPB biogenesis and during VWF exocytosis. A more general application of CLEM as an assay in biological research has the potential to reveal novel cellular, or subcellular, relations between structures that were otherwise unnoticed when studied by light or electron microscopy alone. Future CLEM developments would ideally result in a work-

flow that is easy, fast, reliable and applicable to every biological system. However, the development of such a workflow is challenging and probably impossible as each application will have its own requirements and limitations that are related to the biological question at hand. The large number of currently available CLEM workflows emphasize the versatile application of CLEM. CLEM is used on several scales ranging from locating cells in a block of tissues, to pinpointing molecular or sub-cellular structures at the highest attainable resolution in frozen-hydrated material.

In the field of VWD research, further application of CLEM will be essential to obtain detailed information on the molecular mechanisms that are defective in VWD. To reveal the molecular alterations with electron microscopy it is inevitable to make use of cryo-CLEM in which cryo fluorescence microscopy is combined with cryo electron tomography.[8] To study VWD by cryo-CLEM, blood out-growth endothelial cells (BOECs) cultured from peripheral blood from VWD patients are required. The cellular extremities of the BOECs are probably, similarly as HUVECs, thin enough for the application of whole-cell cryo electron tomography. Previously, it was already shown that these cells can provide a good model for the cellular analysis of VWD[9,10] One major challenge to perform CLEM on these BOECs is to label the WPBs with fluorescent marker. Once the correct transfection technology has been determined, additional live-cell imaging can be performed to, for example, study VWF exocytosis prior to vitrification. After vitrification, cryo fluorescence microscopy is used to locate the specific areas that are suitable for cryo electron tomography.[8] In the setup described above, the imaging of VWF deficiencies upon storage or secretion is limited to the thinner parts of the cell. To study the thicker parts of the cells by cryo electron tomography, a thin (200 – 500 nm) lamellae has to be created by Focused Ion Beam milling to facilitate electron tomography.[11] Although it will be challenging to perform cryo-CLEM on VWD BOECs, we hypothesize that our proposed approach can give the VWD research new perspectives by truly revealing the defective steps on a molecular scale in VWF storage and secretion causing VWD. Additionally our findings may potentially lead to novel treatment strategies.

Apart from the research directed towards the molecular fundamentals of VWD, we also presented electron microscopy techniques that are most suitable to characterize WPBs in vivo in different vascular beds that are found in the vasculature of mammals. Up to date only limited WPB characterization was performed in tissue while increasing evidence suggests large heterogeneity in WPBs between vascu-

lar beds. An electron microscopy technique suitable for imaging pieces of tissue is SBF-SEM. With SBF-SEM large three dimensional data sets can be analyzed to characterize WPB morphology at nanometer scale resolution in the context of tissue and whole cells. For instance, it would be interesting to reveal the WPBs of lung endothelium to assess whether these cells produce the same rounded, potentially Factor VIII containing, WPBs as we observed in endothelial cells in which Factor VIII was introduced upon transduction. Characterization of the WPB distribution in different vascular beds may also contribute to other fields of research and could reveal why certain areas are more prone to thrombosis than others.

In conclusion, the various chapters in this thesis present a variety of imaging techniques using light and electron microscopy. Further application, of the presented and the proposed imaging techniques can provide additional information that may contribute to the understanding of VWD and other vascular diseases. However, to place our finding in a broader perspective it is also essential to combine our image data with data provided by molecular and clinical studies. To facilitate the translation between the different research fields (e.g. clinical, molecular and microscopy research) large accessible image data sets are required in which previous findings are shared. To date, however, access to data that is derived from imaging is limited because it is difficult to quantify visual information in a biological context. This lack of quantification makes it difficult to store image derived data in large, easily accessible databases. By combining imaging techniques to provide not only morphological, but also functional information of the same structure, morphological data can be quantified based on the functional information. Combining imaging techniques to provide not only morphological, but also functional information of the same structure enables quantification of microscopy image data. Techniques such as CLEM will therefore provide the means to make light and electron microscopy image data suitable for data mining and storage in databases for global access today and in the future.

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