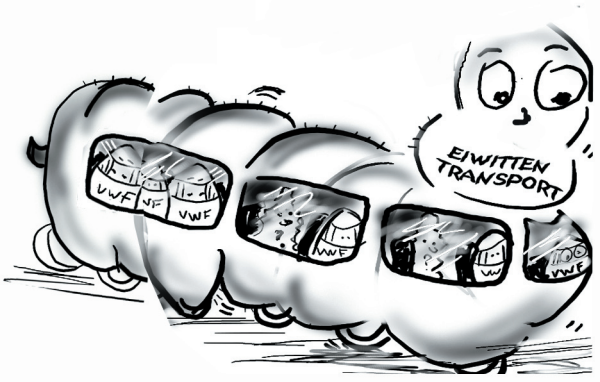
Weibel-Palade bodies

an exciting way out!



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Weibel-Palade bodies
an exciting way out!

Weibel-Palade lichaampjes
een prikkelende uitweg!
(met een samenvatting in het Nederlands)

Proefschrift

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CONTENTS

Chapter 1

GENERAL INTRODUCTION

BIOSYNTHESIS, PROCESSING AND SECRETION OF VON WILLEBRAND FACTOR; BIOLOGICAL IMPLICATIONS

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INTRODUCTION

Von Willebrand factor (VWF) is a multifunctional plasma protein that plays a predominant role in the events that lead to normal arrest of bleeding. VWF is produced by endothelial cells and megakaryocytes and originates from the VWF precursor (pro-VWF), processing of which results in the formation of two large polypeptides, mature VWF and the VWF propeptide (also named VWAgII). Both polypeptides have distinct biological functions. Mature VWF both mediates the adhesion of blood platelets at sites of vascular injury and functions as a stabilizing carrier protein of factor VIII, an essential cofactor of the coagulation system. Its physiological significance is underscored by the well known observations that qualitative or quantitative defects, or von Willebrand's disease, may predispose to a severe bleeding diathesis, both because of defective platelet plug formation and abnormal blood clotting at sites of vascular injury. In addition to its well defined role in platelet adhesion and regulation of the coagulation system, VWF may help to anchor endothelial cells to the extracellular matrix. The propeptide of VWF is required for proper post-translational multimerisation and subsequent storage of VWF. In the last few years, increasing evidence has been obtained that the VWF propeptide might also be involved in a variety of biological processes after it is secreted. It both may modulate platelet function and may act as an inflammatory mediator.

The biosynthesis and the properties of VWF have been reported in comprehensive reviews [1,2]. Here we will briefly discuss the major molecular events associated with its biosynthesis, storage and secretion by endothelial cells. Subsequently, we will focus on the function of VWF as a chaperone protein for factor VIII. In addition, we concentrate on the unusual pleiotropic nature of the VWF propeptide.

BIOSYNTHESIS OF VON WILLEBRAND FACTOR

In the early 1970s, immunohistochemical studies revealed that antibodies to the factor VIII-VWF complex recognized immunoreactive material associated with the endothelium of a variety of human tissues [3,4]. Soon it became clear that endothelial cells from large and smaller veins, capillaries, aorta and arteries indeed synthesize and secrete VWF [5,6]. More recent studies have demonstrated that the VWF gene is differentially expressed in endothelial cells present in different tissues [7]. Besides endothelial cells, also platelets contain VWF produced by the platelet precursor, the megakaryocyte [8,9].

VWF is a typical example of a secretory protein. The molecular events associ-

ated with the biosynthesis and secretion of VWF are, to a large extent similar to the processing steps and routing of many other proteins destined to be externalized. In one respect the biosynthesis of VWF distinguishes itself from the synthesis of most other proteins produced by endothelial cells, in that, at least part of the newly synthesized protein is stored in characteristic organelles, called Weibel-Palade bodies (see below).

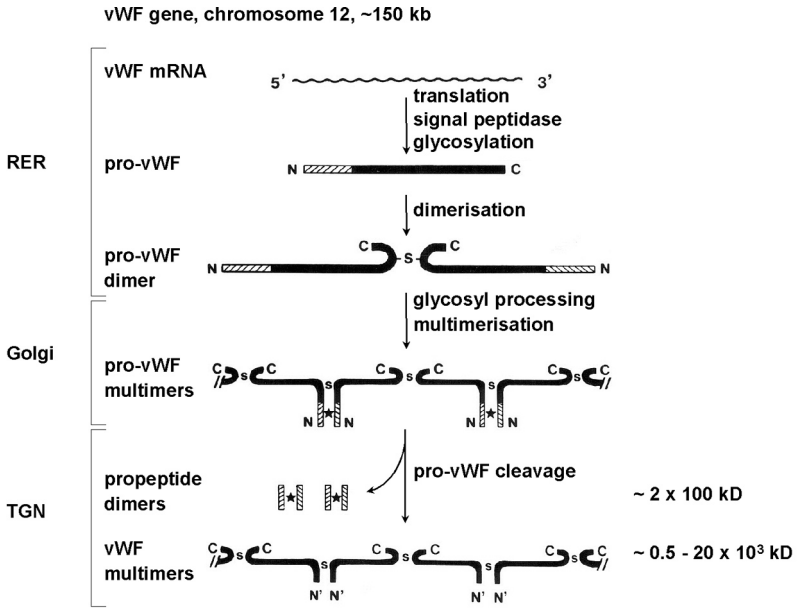

Figure 1. Schematic representation of the processing steps involved in the biosynthesis of VWF. The hatched area represents the propeptide moiety of VWF and the dark area mature VWF. The compartments where the processing steps take place are indicated on the left. H=non-covalent interaction; S=disulfide bonds. The molecular mass of the different VWF species is given on the right. Adapted with permission from [79].

The molecular events associated with the biosynthesis of VWF occur, in terms of time and space, in highly ordered manner (Fig.1). Molecular cloning of the full length human VWF cDNA revealed that the mRNA is translated as a pre-pro-polypeptide, composed of a signal peptide of 22 amino acids, an unusually large pro-peptide of 741 amino acids, and mature a VWF molecule of 2050 amino acid residues. These features indicate that the pre-pro-protein undergoes two proteolytic processing steps before it enters the circulation: cleavage of the signal peptide by a signal peptidase and subsequent cleavage of the propeptide after the arginine residue at position 763[1] by an endoprotease. A remarkable feature of the VWF precursor protein is its highly repetitive structure. The pro-protein is almost entirely (over 90%) composed of four types of repeated domains (A-D) exhibiting internal

[1] Amino acid numbering starts at the initiation codon of the signal peptide

homologies, that are each present in two to five copies (Fig.2).
VWF probably has a complex evolutionary history marked by repeated gene segment duplications and exon shuffling. To a certain extent these domains exert autonomous functions. Roughly speaking a number of distinct binding functions of VWF co-localize with a particular domain. Comparison of the protein sequences revealed that these domains share similarity with distinct, apparently unrelated structural motifs in a number of other proteins with binding functions, including integrins, components of the complement system, thrombospondin and collagens (reviewed in [2]). Also characteristic for the pro-VWF structure is the occurrence of Arg-Gly-Asp (RGD) sequences. One RGD sequence is located near the carboxy-terminal region of the propeptide and one is located near the carboxy-terminal end of the C1 domain. There is ample evidence that the latter RGD sequence is essential for the binding of VWF to platelets [10]. Whether the propeptide RGD sequence plays a similar function has not been established. The RGD motif is not present in the bovine propeptide moiety of pro-VWF, suggesting an insignificant role of this

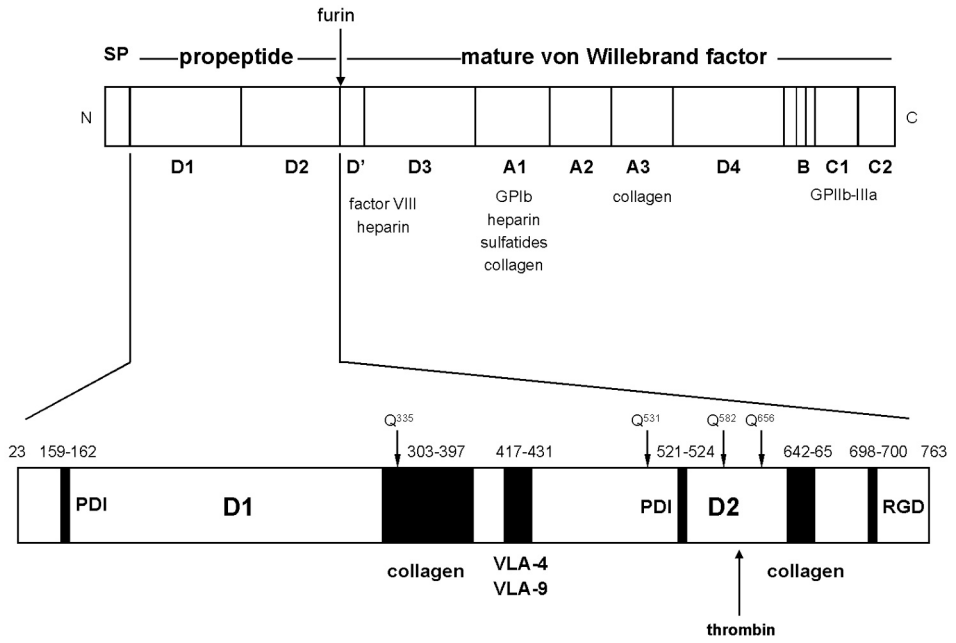

Figure 2. Structure and functional sites of VWF and the VWF propeptide. The upper part represents pre-pro-VWF with its typical domain structure. Pre-pro-VWF (2813 amino acids) is composed of a signal peptide (SP) of 22 amino acids, the VWF propeptide of 741 residues and, at the carboxyl-terminal end, mature VWF of 2050 amino acid residues. The furin cleavage site as well as all known ligand binding sites of mature VWF are schematically depicted in the upper part. The lower part of this figure depicts the primary structure of the VWF propeptide, including PDI and RGD sequences, and its interactive sites. The four Gln (Q) sites of the propeptide of VWF that react with factor XIIIa are indicated by arrows. The numbering of the amino acid residues starts at the signal peptide (SP).

sequence in controlling functional modalities of the propeptide [11].

After cleavage of the signal peptide and translocation in the ER, the primary translation product undergoes N-linked glycosylation and dimerisation (Fig.1) [1]. Dimerisation occurs through disulfide bonds within the carboxy-terminal amino acids of the pro-VWF subunits [12,13]. The pro-VWF dimers are then transported to the Golgi apparatus and post-Golgi compartments, where the high mannose oligosaccharides are processed to the complex form and also sulphation and O-linked glycosylation take place [1,14]. Simultaneously to these post-translational processes, the processed pro-VWF dimers polymerize during the travel through the Golgi compartments. Consecutively, pro-VWF is transported to the trans-Golgi network where further multimerisation and proteolytic cleavage occur, yielding mature VWF multimers and propeptide dimers. After cleavage, VWF and the VWF propeptide remain non-covalently associated, at least within the cell [15].

Detailed analysis of the secondary structure of VWF revealed that inter-subunit disulfide bonds are localized in an amino-terminal region (residues 1046-1458) and carboxy-terminal region (amino acid residues 2671-2813), beyond the domain C2 [13]. Although polymerisation of VWF dimers by formation of intermolecular disulfide bonds located within the D3 domains is apparently not affected by carboxy-terminal disulfide bond formation (and vice versa), polypeptide regions upstream of the domain D3 do control polymerisation. For instance, if the domain D' is deleted or harbors certain point mutations, no multimers are formed [16,17] Similarly, deletion of the propeptide, or missense mutations within the propeptide region, results in defective polymerisation [18-21]. These observations can be interpreted in terms of a "zipper model" which proposes that both propeptide and the domain D' serve to properly align the dimers for inter-dimer crosslinking. Pertinent to this point is the ability of the (cleaved) propeptide to form dimers through non-covalent interactions [22,23].

Accumulated data suggest that the propeptide not only plays a role in positioning the protomers but may also catalyse crosslinking of the D3 domains. Indeed, when mature VWF, devoid of the propeptide, is co-expressed with the free propeptide, each in a separate vector, polymerisation does occur [19]. Moreover, alteration of the primary structure of those peptide regions within the D domains of the propeptide that are highly homologous to the active site of proteins harboring protein disulfide isomerase (PDI) activity, abolishes polymerisation [23]. PDI is the enzyme that catalyses thiol-disulfide interchange reactions in protein substrates leading to disulfide formation and folding of the protein [24]. These observations suggest that the ability of the propeptide to promote interchain disulfide bonding can be ascribed to intrinsic PDI activity of the propeptide. In this respect, the VWF propeptide acts as a intramolecular chaperone protein [25].

The polymerisation process is accompanied by cleavage of the dimer form of the precursor into mature VWF and the free propeptide [26]. The propeptide cleavage occurs at a dibasic site, one of the most common processing motifs in mammalian pro-proteins and prohormones [27]. Two members of the eukaryotic subtilisin-like protease family, furin (also called PACE) and PACE4, were shown to process pro-VWF to its mature form [28,29]. These proteases share the same cleavage site but have different substrate specifcities [30]. Most likely, furin is the enzyme responsible for pro-VWF cleavage within the endothelium [31,32].

STORAGE OF VWF BY ENDOTHELIAL CELLS IN WEIBEL-PALADE BODIES

VWF distinguishes itself from many other endothelial proteins in that it can be secreted from the cell by more than one pathway, the constitutive and the regulated pathway (Fig.3). The constitutive secretory nature of the endothelium is reflected by the observation that soon after synthesis, VWF accumulates extracellularly in the absence of a stimulus [33]. On the other hand, if endothelial cells are exposed to agonists that give rise to an increase of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$), such as thrombin, histamine or the calcium ionophore A23187, or to cAMP-raising agents, such as epinephrine or forskolin, VWF is rapidly secreted by the cell [34-36]. The rate of

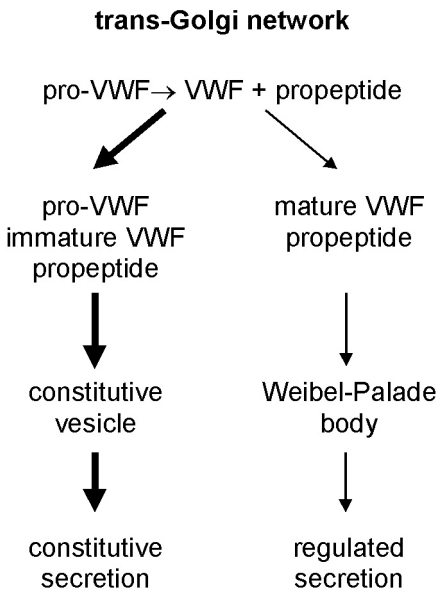

Figure 3. Partitioning of VWF, propeptide and pro-VWF between the constitutive and regulated secretory pathway by vascular endothelial cells. The pro-VWF molecule is processed in the trans-Golgi network and results in heterogenous forms of VWF, ranging from dimers to large polymers. The largest proportion of the VWF species, consisting of partially processed and inmature VWF, is secreted through the constitutive pathway. The remainder, the largest, fully processed and functional VWF, is sorted into Weibel-Palade bodies and secreted via the regulated pathway. This model proposes that the majority of VWF is secreted through the constitutive pathway [48]

this type of secretion is much higher than the biosynthetic rate of VWF. The stimulus-induced secretion of VWF originates from storage vesicles, previously identified as Weibel-Palade bodies [37]. These vesicles are endothelial cell-specific elongated organelles, enclosed by a limiting membrane, which are about 0.1 μm wide and up to 4 μm long [38]. Besides VWF, Weibel-Palade bodies have been shown to contain a subset of other proteins, including P-selectin and endothelin (Table 1). Similar to VWF, these proteins can be secreted upon demand by endothelial cells upon stimulation by Ca^{2+}- or cAMP agonists. Apparently, Weibel-Palade bodies are not only restricted for VWF storage.

Table 1. Residents of Weibel-Palade bodies

	References
Mature vWF	[81,82]
VWF propeptide	[82,83]
P-selectin	[84-86]
CD63	[87]
Endothelin	[88]
Interleukin-8	[89,90]
α 1,3-fucosyltransferase VI	[91]
Tissue-type plasminogen activator	[92,93]

Electron microscopical studies revealed that Weibel-Palade bodies most likely originate from the trans-Golgi network [39]. Immunostaining of ultrathin frozen sections of cultured endothelial cells revealed that, together with apparently mature Weibel-Palade bodies, densely packed VWF, surrounded by a limiting membrane, is occasionally found in close apposition with the trans-Golgi network [40]. This observation supports the view that VWF-containing storage vesicles emerge through budding from the Golgi complex, an event generally associated with the secretion of proteins in eukaryotes [41]. It has been proposed that targeting of VWF to Weibel-Palade bodies occurs as a consequence of selective aggregation, by means of multimerisation of this protein in the trans-Golgi network [40,42]. However, evidence has been obtained that VWF-multimerisation is not the sole targeting signal. It has been proposed that the propeptide could also play a role in this process [23,43]. Apparently, the sorting mechanism is more complex than previously understood.

VON WILLEBRAND SECRETION AND CATABOLISM

Regulated secretion of VWF involves the translocation of Weibel-Palade bodies from the cytoplasm towards the plasma membrane and fusion of these vesicles with the plasma membrane. These events are regulated by second messengers in response to $[Ca^{2+}]_i$ - or cAMP raising agents. There is ample evidence that G proteins are involved in the $[Ca^{2+}]_i$-mediated VWF secretion by endothelial cells (Fig.4) [44-46].

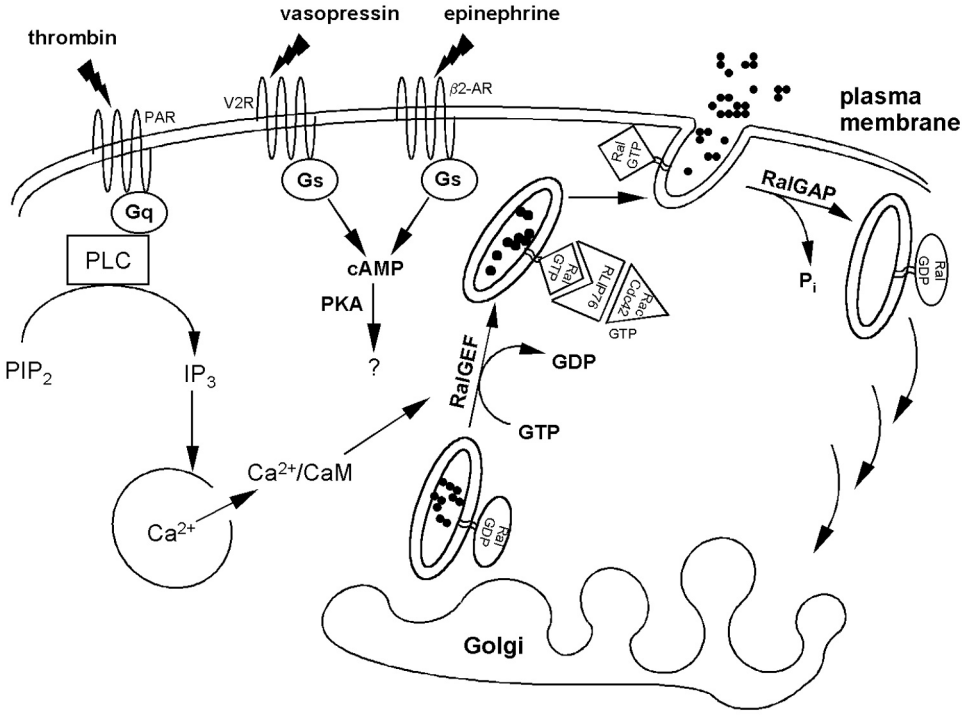

Figure 4. Machinery of stimulus-induced exocytosis of Weibel-Palade bodies by endothelial cells. Upon stimulation of endothelial cells by agonists such as thrombin, the phosphatidylinositol pathway is activated, resulting in a rise of cytoplasmic Ca^{2+}. Thrombin signalling is mediated by a G protein-coupled protease-activated receptor (PAR). Calcium in complex with calmodulin (CaM) binds the small GTP-binding protein Ral in its GDP-form, a small amount of which is associated with the Weibel-Palade bodies. GDP on Ral is then exchanged with for GTP by mediation of an Ral guanine nucleotide exchange factor (RalGEF). Ral in its GTP-bound conformation interacts downstream with effector molecules such as RLIP76, a GTPase activating protein for Cdc42 and Rac. The hydrolysis of GTP on Cdc42 or Rac may result in cytoskeleton rearrangement, which may precede exocytosis of Weibel-Palade bodies. The contents of Weibel-Palade bodies are released into the circulation (e.g. VWF, black circles) or exposed at the plasma membrane (e.g. P-selectin). Regulated, receptor-mediated secretion of VWF can also be accomplished by agonists, such as epinephrine or DDAVP (1-deamino-8-D-arginine vasopressin), agents known for their ability to activate G protein-coupled receptors and cAMP-dependent signalling, independently of a rise of cytosolic Ca^{2+}. The molecular mechanism of cAMP-mediated VWF secretion have not been identified. V2R: vasopressin type 2 receptor, ß2-AR: ß2-adrenergic receptor. (Courtesy of dr. Hubert de Leeuw [80]).

However their role in cAMP-dependent exocytosis remains to be established. Qualitative differences in Weibel-Palade body secretion are observed in response to $[Ca^{2+}]_i$ - and cAMP agonists [46]. $[Ca^{2+}]_i$ induces release of both peripheral and central granules, whereas cAMP involves only vesicles located in the periphery of the cell. It is worth noting that the role of 1-deamino-8-D-arginine vasopressin (DDAVP), which is widely used to raise plasma VWF and factor VIII levels in patients with von Willebrand's disease and haemophilia A, has recently been delineated [47]. It has been shown that DDAVP can directly induce VWF secretion from human microvascular endothelial cells by activation of the vasopressin V2 receptor (V2R). This process is mediated by a rise in cAMP.

PHYSIOLOGICAL SIGNIFICANCE OF VWF PROCESSING AND SECRETION.

As outlined above, expression of the VWF gene is associated with a number of post-translational modifications, including a discrete endoproteolytic cleavage step. The latter step results in the generation of mature VWF and the propeptide moiety of pro-VWF. Subsequently these polypeptides are released to the outside of the cell, either directly or after appropriate stimulation of the cell. After release, VWF and its propeptide have divergent fates. VWF serves a role in controlling platelet adhesion and aggregation and acts as a chaperone protein for factor VIII; the propeptide may act as an antagonist of platelet function and may serve a role as an inflammatory mediator. Little is known about the physiological significance of the diversity of processing, secretion and the ability of endothelial cells to store VWF. It seems reasonable to assume that a subset of biologically active VWF polymers become readily available in the vicinity of the injured vessel upon activation of endothelial cells and subsequent exocytosis of Weibel-Palade bodies (for instance induced by thrombin formed locally as a result of vascular damage). In particular these VWF species play a prominent role in controlling platelet adhesion and aggregation (and possibly controlling factor VIII function, see below). Because of its limited degree of polymerisation, VWF released through the constitutive pathway would be less efficient in this respect. In vitro studies have shown that a significant portion of de novo synthesised VWF is secreted through the constitutive pathway as partially processed, incompletely polymerised and functionally incompetent protein [48]. Only fully processed, substantially polymerised and functionally mature VWF is stored in the Weibel-Palade bodies, together with its propeptide (Fig.3). To what extent the plasma VWF pool stems from the constitutive route in vivo, is unknown. Only trace amounts of pro-VWF are detectable in plasma [49]. This

suggests that under normal physiological conditions the constitutive pathway does not substantially contribute to the release of VWF. On the other hand, it is possible that after release pro-VWF is rapidly cleared from the circulation or, alternatively, converted into mature VWF and propeptide outside the cell. Indeed, animal experimental studies have shown that upon infusion of (unprocessed) pro-VWF the VWF propeptide is rapidly cleaved from its precursor [50]. It remains unclear, therefore, to what extent release through the constitutive pathway plays a role in the replenishment of the plasma VWF pool. In any case, only mature VWF and its propeptide are detectable in plasma in significant amounts (50 and 5 nM respectively), not their precursor, pro-VWF [49].

FUNCTIONS OF VWF

After its release from the endothelium (and platelets) VWF serves two essential biological functions: it mediates the adhesion and aggregation of platelets at sites of vascular injury and modulates the survival and function of factor VIII. In addition, proper haemodynamic conditions (high shear rate) and highly polymerised VWF molecules are required to ascertain efficient platelet adhesion and aggregation. The molecular basis and physical chemical backgrounds of events associated with VWF-mediated platelet functions are well documented (reviewed in [2] and [10]). The factor VIII chaperone function of VWF is less well understood and this unique property will be discussed here.

Factor VIII binding

VWF circulates in plasma with factor VIII as a non-covalent complex. This interaction extends the survival of factor VIII in the circulation. Any change in plasma VWF level is coupled with a concordant change in the plasma concentration of factor VIII. For instance, low VWF levels, as in von Willebrand's disease, are associated with low plasma factor VIII levels as well. Similarly, qualitative VWF defects, as in von Willebrand's disease type 2N (see below), may abolish proper factor VIII-VWF interactions and, as a consequence, plasma factor VIII levels are decreased. In clinical conditions associated with elevated VWF levels, such as malignancies, sepsis or liver disease, factor VIII levels seem to increase in a coordinate fashion [51,52]. Similarly, also physiological stimuli such as exercise or pregnancy enhance VWF levels with a concomitant rise in factor VIII levels. These examples clearly illustrate that VWF is essential for the stabilisation of factor VIII in the circulation. The opposite is not the case; plasma VWF levels are not influenced by factor VIII levels.

The molecular basis of the factor VIII-VWF complex formation is fairly well

understood, at least the polypeptide domains and interactive sites of the VWF and factor VIII molecule that mutually interact have been identified (reviewed in [53]). The amino-terminal D' domain of VWF comprises the primary factor VIII binding site [54]. Only in its dimeric form this Cys-rich domain is able to bind factor VIII, indicating a complex mode of protein-protein interaction. In patients with a markedly decreased affinity of their VWF with (otherwise normal) factor VIII, (socalled von Willebrand's disease type 2N) several missense mutations in VWF have been identified that selectively abolish factor VIII binding [55,56]. The majority of mutations were localized to the D' domain. In most (though not all) cases reported these mutations do not affect the plasma VWF levels or the reactivity of VWF with platelets or the subendothelial connective tissue. Only the affinity for factor VIII is decreased. Recent studies indicate that multiple amino acid residues within the D' domain are critical in stabilising the conformational structure of this domain and, as a consequence, factor VIII binding [17,57]. The complementary interactive sites for VWF on the factor VIII molecule have also been identified (reviewed in [53]).

VWF not only binds to factor VIII and thereby controls its biological survival, it also protects factor VIII from premature proteolytic cleavages and inactivation. Possibly biological survival and proteolytic degradation are interconnected, though a causal relationship has not been identified. VWF and factor VIII are probably synthesized by different cell types [53] and during or after release into the circulation these proteins form a tight, non-covalent complex. Each VWF monomer is able to bind one factor VIII molecule. However, in *vivo* not all VWF monomers are bound to factor VIII. Rather a relatively small portion (1-2%) of the available VWF monomers is occupied by factor VIII [58]. These complexes are relatively stable. Only upon triggering of the coagulation system one of the VWF-binding sites on the factor VIII molecule is cleaved off, notably by thrombin, resulting in dissociation of the complex and subsequent conversion of factor VIII into its active configuration (factor VIIIa) [59].

VWF modulates the function of factor VIII at various levels. It inhibits the interaction of factor VIII to different proteases of the coagulation system, including factor IX, factor X, protein C as well as negatively charged phospholipid membranes, thereby preventing premature activation of the coagulation system. These interactions most likely play a significant role in controlling haemostatic responses to vascular injury. There is indeed ample evidence that VWF can protect factor VIII against proteolytic degradation, at least *in vitro* (reviewed in [53]). It seems reasonable to assume, therefore, that the decreased half-life of factor VIII in the absence of VWF is due to premature cleavages of factor VIII and subsequent enhanced clearance of the proteolytically modified factor VIII molecule. However, experimental data in support of this view is lacking so far.

Recent reports have shown that VWF could also control the biological half-life of factor VIII by controlling its interaction with LRP (low density lipoprotein receptor-related protein) [60]. LRP is a multifunctional scavenger receptor involved in the clearance of a spectrum of ligands, including proteases, protease-inhibitor complexes, lipases and apolipoproteins from the circulation (reviewed in [61,62]). LRP also binds factor VIII and thereby mediates its cellular internalisation and degradation [63,64]. These processes are inhibited by VWF. These observations suggest that VWF may also control factor VIII clearance by a mechanism independent of premature proteolytic breakdown of factor VIII. Indeed, in VWF deficient mice the half-life of factor VIII is prolonged by pre-infusion of the animals with RAP, a LRP antagonist [60]. Future studies should reveal the biological significance of the latter clearance mechanism.

Functions of VWF-propeptide

As discussed above, the VWF-propeptide plays an important role in controlling intracellular targeting and polymerisation of VWF. During the maturation of VWF, the propeptide is subsequently cleaved from pro-VWF but is co-stored in the Weibel-Palade bodies and co-secreted with mature VWF in response to stimulation of endothelial cells rather than being secreted or degraded as a waste product. Indeed, plasma levels of propeptide (and VWF) are markedly increased under clinical conditions known to be associated with perturbation of the endothelium, such as septicaemia or thrombotic thrombocytopenic purpura (TTP) [65]. It has been thought that, after its release, the propeptide is biologically inert. This is most likely not the case. Accumulating evidence suggests that the propeptide, apart from its presumed PDI activity (see above), possesses a number of functions that could serve a role in controlling inflammatory and cell adhesion processes. Recent studies indicated that the propeptide harbours a number of sites that interact with constituents of the connective tissue of the subendothelium as well as integrins (Table 2).

Evidence has been obtained that the VWF propeptide specifically binds to collagen type I and is able to inhibit collagen-induced platelet aggregation [66,67]. The latter effect is probably due to inhibition of the interaction between collagen and the collagen receptor. Detailed analysis of the ability of different monoclonal anti-propeptide antibodies to interfere with the binding of VWF propeptide to collagen revealed that the propeptide contains at least two collagen-binding sites [68] (See Figure 2 above). One of these peptide domains is located in the first Cys-rich region of the propeptide molecule (Glu^{303}-Lys^{397}). The second collagen-binding site is located in a Cys-rich region near the carboxy-terminal region of the VWF propeptide, extending from residues Phe^{592} to Lys^{704}. In particular, the sequence WREPSFCALS (Trp^{642}-Ser^{651}) is involved in collagen binding [69]. Although the VWF propep-

Table 2. Von Willebrand factor gene products

	function	ligands	References
Pro-VWF	targeting	P-selectin	[94]
Mature VWF	haemostasis	Collagen	[2]
		GPIb, GPIIb-IIIa	[10]
		Factor VIII	[53]
VWF propeptide	PDI*	VWF	[23]
	targeting	VWF	[23,43]
	inflammatory mediator	Collagen	[66-69]
		Factor XIIIa	[73,74]
		VLA-4, VLA-9	[75-77]

* PDI, protein disulfide isomerase

tide preferentially binds to native type I collagen fibrils, the Phe^{592}-Lys^{704} fragment and the decapeptide bind to several other collagens, both in the native as well as in a heat-denatured state. Also mature VWF harbours collagen binding sites (Fig.2). Peptides regions involved in the latter are structurally distinct from the propeptide binding sites [69,70].

The VWF propeptide has also been shown to be present on the surface of platelets [71]. As antibodies to the propeptide may induce platelet activation and aggregation, it has been suggested that membrane-bound propeptide could serve a role in controlling platelet function [72]. The biological significance of the propeptide as a modulator of platelet function remains to be established, however.

A few reports demonstrated that the VWF propeptide is a substrate for coagulation factor XIIIa. This transglutaminase is able to catalyse the cross-linking of the propeptide to laminin, a constituent of the extracellular matrix [73,74]. The Gln residues in the propeptide specifically reacting with factor XIIIa as amine acceptor have been identified (Fig.2) [74]. Only 4 out of the 40 Gln residues of the propeptide act in this manner, highlighting the specificity of the factor XIIIa-propeptide interaction. As laminin has a variety of cellular functions, including its role as cell attachment or cell migration mediator, it is tempting to speculate that the propeptide could play a role in modulating these processes.

Of particular interest is the observation that the propeptide is able to promote specific adhesion and spreading of certain cell types, in a integrin-dependant manner [75-77] These processes are mediated by the α4ß1 (VLA4) and the α9ß1

(VLA9) integrins [76,77]. These integrins bind to the same propeptide site, involving residues 417-432. Interestingly, these integrins also bind factor XIIIa, the enzyme that catalyses the cross-linking of the propeptide to laminin. Both integrins are highly expressed on leukocytes; VLA-4 is primarily expressed by lymphocytes, monocytes and eosinophils, whereas VLA-9 is mostly found on neutrophils [78]. As both integrins play a prominent role in transendothelial migration of these cells, the propeptide could modulate these processes.

Taken together, in addition to its important intracellular function, evidence is accumulating that the propeptide serves a number of extracellular functions, at least in vitro. Future studies should reveal the physiological and pathophysiological significance of these observations.

SCOPE OF THIS THESIS

As outlined above, endothelial cells are able to secrete VWF either constitutively or through the regulated pathway. As illustrated in Figure 5, storage of VWF in Weibel-Palade bodies is one of the essential steps for its rapid regulated secretion in circulation, and its subsequent function in correct haemostasis. Interestingly, Weibel-Palade bodies are not only restricted to VWF-storage, but are also capable to store and secrete proteins with divergent extracellular functions, among which the VWF-propeptide, P-selectin and IL-8. However, mechanisms such as sorting of proteins to Weibel-Palade bodies, Weibel-Palade body trafficking in the cell and secretion of their content in circulation are far of being understood. Each chapter in this thesis addresses an issue involved in the life cycle of Weibel-Palade bodies or the extracellular function of one of their constituents. Figure 5 illustrates the different steps that were studied in this thesis:

1. targeting of proteins into Weibel-Palade bodies
2. intracellular trafficking of Weibel-Palade bodies
3. exocytosis
4. extracellular function of the VWF-propeptide, a Weibel-Palade body constituent.

The mechanism of protein targeting and in particular the targeting of IL-8 into Weibel-Palade bodies, and the role of VWF in this mechanism is studied in Chapter 2. Chapter 3 focuses on the trafficking of Weibel-Palade bodies inside the cell and their secretory behaviour upon stimulation of the cell. Weibel-Palade body exocytosis was also investigated in Chapter 4, focussing on the role of a small GTP binding protein Ral in this process.

The second part of this thesis concentrates on one of the Weibel-Palade body

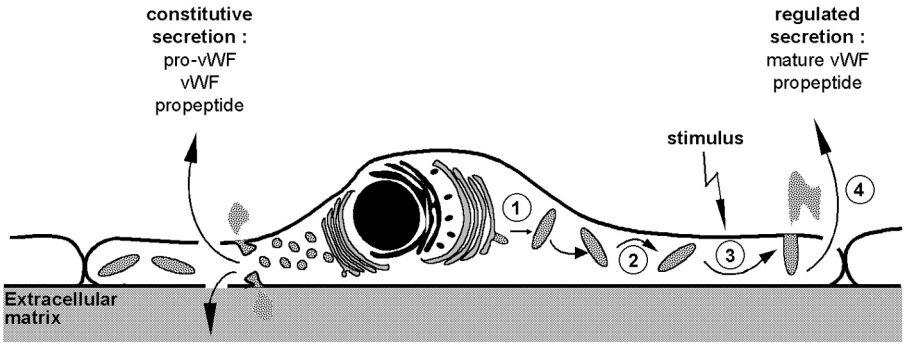

Figure 5. Schematic representation of the two secretion pathways in endothelial cells and the processes studied in this thesis. VWF and its propeptide can be released either through the constitutive or the regulated pathway. Prior to its regulated secretion VWF is stored in Weibel-Palade bodies (indicated as rod-shaped organelles). Upon stimulation of the cell, Weibel-Palade bodies release their content extracellularly. Numbers indicate the processes investigated in this thesis: **1.**targeting and storage of proteins into Weibel-Palade bodies **2.**intracellular trafficking of Weibel-Palade bodies **3.**exocytosis **4.**extracellular function of the VWF-propeptide, a Weibel-Palade body constituent.

constituent, the VWF-propeptide. In contrast to its well-defined role in controlling intracellular targeting and polymerisation of VWF, less evidence has been provided regarding its biological functions after its release from the cell. Chapter 5, a literature review about the VWF-propeptide, describes in more detail the functions of the propeptide in vascular disorders so far known, including its role as a marker for endothelium perturbation. The latter property of VWF-propeptide served to examine the degree endothelial cell stimulation in patients suffering of thrombotic thrombocytopenic purpura (chapter 6). Chapter 7 focuses on another property of the propeptide, namely its possible role as an inflammatory mediator.

The implications of our findings for the unravelling of the life cycle of Weibel-Palade bodies are discussed in Chapter 8 and directions for future research are indicated.

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Chapter 2

VON WILLEBRAND FACTOR TARGETS IL-8 TO WEIBEL-PALADE BODIES IN AN ENDOTHELIAL CELL LINE

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ABSTRACT

Vascular endothelial cells are able to store the chemotactic cytokine interleukin-8 (IL-8) in specialized storage vesicles, Weibel-Palade bodies, together with von Willebrand factor (VWF) and P-selectin. We investigated whether VWF plays a role in the sorting of IL-8 into these organelles. We examined the effect of VWF expression on IL-8 targeting in an endothelial cell line (EC-RF24). This cell line has retained the typical phenotypic characteristics of primary endothelial cells but has lost the capacity to produce VWF in appreciable amounts. EC-RF24 cells were retrovirally transduced with a vector encoding a VWF-green fluorescent protein chimera (VWF-GFP). This approach enables direct visualization of the cellular distribution and secretory behavior of the VWF-GFP hybrid. Expression of VWF-GFP resulted in the generation of Weibel-Palade body-like organelles as shown by the co-localization of VWF-GFP and P-selectin. VWF-GFP expressing EC-RF24 cells also showed significant co-localization of VWF-GFP with IL-8 in these storage vesicles. Live cell imaging revealed that the number of VWF-GFP containg granules decreased upon cell stimulation. These observations indicate that VWF plays an active role in sequestering IL-8 into Weibel-Palade bodies.

INTRODUCTION

Von Willebrand factor (VWF) is a multifunctional plasma protein that is required for normal haemostasis. Its physiological significance is demonstrated by the observation that VWF deficiency, or von Willebrand's disease, may predispose to a severe bleeding tendency. VWF is synthesized by endothelial cells and megakaryocytes and plays a major role in platelet adhesion and haemostatic plug formation at sites of vascular injury. In endothelial cells VWF is secreted through two different pathways. The major portion of de novo synthesized VWF is secreted constitutively. The remainder is stored in specialized vesicles, the so-called Weibel-Palade bodies, and is secreted only upon stimulation of the cell by Ca^{2+}- and/or cAMP-raising agents. Only fully processed, haemostatically active VWF is stored in Weibel-Palade bodies.

In addition to VWF, these secretory granules also contain a number of other proteins with different biological functions, including P-selectin [1], CD63 [2], endothelin [3], IL-8 [4;5], tissue-type plasminogen activator [6;7] and α1,3-fucosyltransferase VI [8]. The mechanism responsible for sorting of these proteins into these secretory vesicles is poorly understood. Accumulated evidence suggests that VWF not only plays a role in directing the formation of Weibel-Palade bodies [9-11], but

may also have a helper function in the sorting of distinct proteins to Weibel-Palade bodies [11;12]. For instance, when an epithelial cell line, which synthesizes P-selectin but does not retain this receptor in storage vesicles, was transfected with VWF-cDNA, Weibel-Palade body-like organelles, containing both VWF and P-selectin, were formed [11]. Similarly, in VWF-deficient mice, P-selectin is not properly stored in endothelial cells [12]. These observations suggest that VWF may trigger sequestering of structurally unrelated proteins into Weibel-Palade bodies. To further substantiate this hypothesis we have studied the role of VWF in controlling the sorting of IL-8 into Weibel-Palade bodies. For this purpose we used a human endothelial cell line (EC-RF24, [13]) with markedly reduced VWF synthesis and decreased Weibel-Palade bodies. Preliminary experiments showed that this cell line is not capable of storing IL-8 in significant amounts. We used this cell line as a model system to address the hypothesis outlined above. Here, we demonstrate that when VWF synthesis is restored in these cells by means of retroviral transduction, IL-8 together with VWF and P-selectin is targeted into granules that, in terms of morphology and secretory behavior, are similar to Weibel-Palade bodies.

MATERIALS AND METHODS

Cell culture

Freshly isolated human umbilical vein endothelial cells (HUVEC) [14] and the immortalized HUVEC cell line EC-RF24 [13] were cultured in medium containing an equal mixture of RPMI 1640 and Medium 199 (Gibco BRL, Paisley, U.K.) supplemented with 20% human serum, 2 mM glutamine (Merck, Darmstadt, Germany), 100 units/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml amphotericin B (Gibco) [14]. HUVEC were used for all experiments after their fourth passage. For real-time experiments, EC-RF24 transfected cells were grown in 35 mm-diameter gelatin-coated glass coverslips and were used at confluency (4×10^4 cells per cm^2).

Immunofluorescence staining and microscopy

For immunofluorescence studies, cells were grown on 1 cm-diameter gelatin-coated glass coverslips. To enhance IL-8 synthesis, confluent cells were pre-stimulated for 24 h at 37°C with 10 ng/ml IL-1β (Strathmann Biotech GMBH, Hannover, Germany) containing culture medium. Subsequently, cells were fixed in 3.7% formaldehyde and permeabilized with a PBS buffer containing 0.02% saponine (Sigma) and 1% bovine serum albumin. The cells were then incubated with unconjugated primary antibodies, washed three times, and incubated with conjugated secondary antibodies, as indicated in the figure legends. Cells were embedded in Vectashield

mounting medium (Vecta Laboratories, Burlington, CA, USA) and viewed by confocal microscopy using a Zeiss LSM510 (Carl Zeiss, Heidelberg, Germany). Confocal images were recorded using standard settings for GFP and Texas-Red detection. When appropriate, images were recorded in the Multi-track mode (sequential scanning) to exclude interchannel crosstalk.

Antibodies

VWF was detected by immunofluorescence with a monoclonal antibody (CLB-RAg 35, CLB, Amsterdam, The Netherlands) directed against the A1 domain of VWF [15]. A rabbit anti-human cathepsin D polyclonal antiserum, a generous gift from Dr. J.M.F.G. Aerts, Department of Biochemistry, Academic Medical Center, Amsterdam, The Netherlands, was used as a lysosomal marker. The monoclonal antibody to P-selectin (RUU 1.18) was kindly provided by Dr H. K. Nieuwenhuis, University Medical Center Utrecht, Utrecht, The Netherlands [16]. As secondary antibody, we used either Texas-red-, FITC-conjugated goat-anti-mouse IgG or horse-anti-rabbit IgG (CLB). For IL-8 detection, a biotinylated polyclonal sheep anti-human IL-8 antiserum was used (CLB), followed by poly-HRP-labeled, FITC-labeled or Alexa594-labeled streptavidin (Molecular Probes, Eugene, Oregon, U.S.A).

Construction of the LZRS IRES VWF-GFP vector

VWF-GFP was constructed by replacing the VWF A2 domain by the sequence encoding the green fluorescent protein (GFP). The GFP fragment was amplified from the pEGFP-N3 vector by PCR using primers containing the XmaI and BsPI restriction sites flanking the A2 domain and the ends of the GFP coding sequence. The primers used were as follows: GFP-sense: 5'-AATCCCGGGGGTGAGCAAGGGCGAGGAG-3'; GFP-antisense: 5'-AATTCCGGACTTGTACAGCTCGTCCAT-3'. A recombinant retroviral vector for delivery of the VWF-GFP gene was constructed using the dicistronic retroviral LZRS vector as described previously [17]. The fragment was inserted downstream of the 5' long terminal repeat (LTR) using EcoRI restriction site.

Retroviral gene transduction

Helper-free recombinant retrovirus was produced after transfection of the LZRS-VWF-GFP DNA into Phoenix-A cells (a 293T-based amphotropic retroviral packaging cell line) [18] by calcium phosphate transfection (Gibco). The cells were cultured in medium containing IMDM, 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 µg /ml streptomycin (all from Gibco) and 2 µg/ml puromycin (Sigma). For the production of virus, cells were maintained for at least 6 h in medium lacking puromycin. EC-RF24 cells, grown on 35 mm-diameter fibronectin-coated wells until

a confluency of 20-40%, were transduced with the harvested virus supernatant as previously described [19]. The transduction efficiency was tested by determining the percentage of GFP positive cells using flow cytometry analysis with a FACSscan (Becton Dickinson, San Jose, CA, USA).

Multimer analysis

EC-RF24-VWF-GFP were grown in fibronectin coated 80 cm^2 flasks. At confluence, cells were incubated for 60 min in 6 ml serum-free medium containing 1U/ml thrombin (Sigma). Cell supernatants were collected and cleared of cell debris by centrifugation. The medium containing VWF was concentrated by incubation with 0.5 volume of buffer containing 50 mM imidazol pH 6.5, 100 mM NaCl, 10 mM benzamidine and 1% (v/v) S-Sepharose Fast Flow (Pharmacia; Uppsala, Sweden) for 20 h. S-Sepharose was collected and suspended in sample buffer (9.7 M urea, 4 ml 10% SDS, 1 ml 0.5% bromophenol blue and 5 ml stacking gel buffer, pH 6.7 containing 70 mM Tris, 4 mM Na_2EDTA and 0.4% SDS) and boiled for 5 min. Samples (5-10 ng VWF) were layered on a 1.4% SeaKem HGT (P) agarose gel (BioWhittaker Molecular Applications; Rockland, ME USA), run for 2 h at 100 V and 45 mA and blotted on a polyvinylidene difluoride filter (Immobilon-P transfer membranes; Millipore Corporations, Bedford). Finally, blots were incubated with anti-GFP (PoAb A11122, Molecular Probes, Eugene, Oregon, U.S.A) and HRP-labeled horse-anti-rabbit antibodies or HRP-labeled anti-VWF (DAKO, Denmark A/S, Glostrup, Denmark) polyclonal antibodies. Multimers were visualized by ECL (Roche Diagnostics Nederland B.V., Almere, The Netherlands).

Quantitative analysis of Weibel-Palade bodies in living cells

EC-RF24-VWF-GFP were grown on 10 mm-diameter gelatin-coated glass coverslips. At confluency, cells were mounted in an incubation chamber (LaCon, Steinberg, Baden-Württemberg, Germany) in HEPES medium consisting of 20 mM Hepes, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 1.2 mM K_2HPO_4, that was supplemented with 1 mg/ml glucose and 1% human serum albumin. Confocal images were recorded with a Zeiss LSM510 using standard settings for GFP. During analysis, the sample chamber was maintained at 37^{o}C using a thermostatted chamber holder. Selected cells were first imaged for 10 min in their initial medium. Subsequently, the medium was replaced by medium with or without 50 ng/ml PMA (Sigma Chemical Co., St Louis, Mo., U.S.A.). Images of VWF-GFP containing granules in living EC-RF24-VWF-GFP were generated by making series of optical sections (Z-stacks, 400 nm interval/thickness) of single cells at different time points. Three-dimensional analysis (x-y-z axis) was performed using depth-coding software (application for Zeiss LSM510, version 2.3) that allowed the monitoring

of all Weibel-Palade bodies present in each cell. The total number of vesicles per cell was counted before and 60 minutes after stimulation with PMA. The difference between the number of Weibel-Palade bodies counted at 0 and 60 minutes was analysed by the paired Student's t-test. The difference between the number of Weibel-Palade bodies in PMA-stimulated cells versus control cells was analysed using the umpaired Student's t-test.

RESULTS

Co-localization of VWF and IL-8 in HUVEC.

It has been previously shown that IL-8 accumulates in Weibel-Palade bodies after prolonged stimulation of endothelial cells and co-localizes with VWF [4;5]. This feature was further explored to study our hypothesis that sorting of IL-8 to these secretory vesicles is directed by VWF. Staining of unstimulated HUVEC with Texas-red-conjugated anti-VWF demonstrated VWF in the rod-shaped vesicles corresponding to Weibel-Palade bodies (Fig.1a,c). These cells did not stain with FITC-conjugated anti-IL-8. However, upon de novo synthesis induced by IL-1β, cells did stain with anti-IL8 antibody (Fig.1e,f). IL-8 was observed both in structures

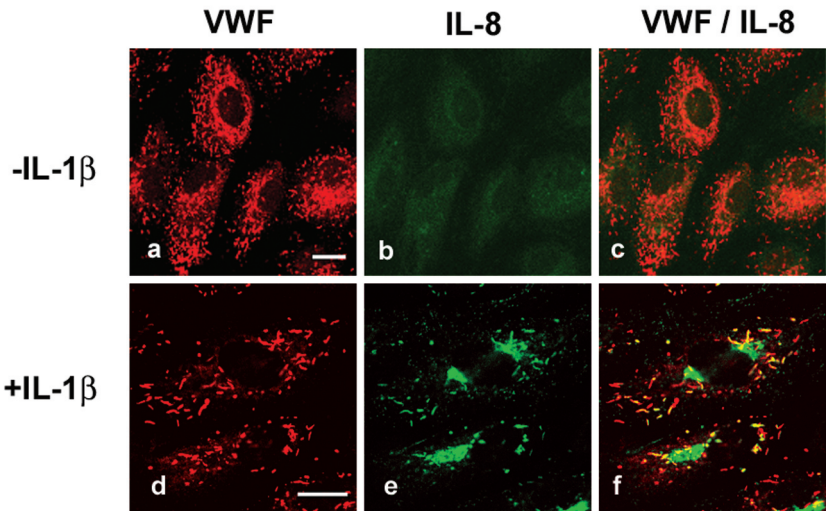

Figure 1. VWF and IL-8 localization in cultured HUVEC. Untreated endothelial cells (**a-c**) or cells treated for 24 h with 10 ng/ml IL-1β (**d-f**), were immunostained for VWF (**a,d**), IL-8 (**b,e**) or both (**c,f**). VWF (red) was stained with the monoclonal anti-VWF CLB-Rag 35 and Texas-red-conjugated goat anti-mouse IgG. IL-8, in green, was stained with biotinylated polyclonal anti-IL-8 antibodies and FITC-conjugated streptavidin. Overlapping distribution of VWF and IL-8 is shown in yellow (**c,f**). Scale bars, 10 μm.

typical of the Golgi apparatus and rod-shaped vesicles (Fig.1e). The latter were similar to those containing VWF (Fig.1d,f). Some vesicles stained for VWF only. We did not find granules that stained only for IL-8 (Fig.1f). Similarly, HUVEC stimulated with endotoxin, instead of IL-1β, revealed granular co-localization of IL-8 and VWF (data not shown).

VWF and IL-8 expression by EC-RF24 cells

To determine whether co-localization of IL-8 and VWF is mediated by VWF expression, we studied the subcellular localization and secretion of IL-8 by immortalized, VWF-deficient endothelial cells (EC-RF24). These cells originated from wild-type HUVEC and have retained several phenotypic characteristics of HUVEC [13]. However, their VWF expression level has decreased dramatically upon culture passages [13]. We took advantage of this phenotype to investigate whether this cell line could be used as a model to study VWF-mediated sorting of IL-8. EC-RF24 cells were cultured for 24 h in medium with or without IL-1β and were subsequently stained for VWF and IL-8 with the appropriate antibodies. Compared to HUVEC (Fig.1a), untreated EC-RF24 demonstrated significant reduced vesicular staining for VWF (Fig.2a,c). IL-8 was sporadically detected in these cells (Fig.2b,c). When EC-RF24 cells were pre-treated with IL-1β, VWF staining was virtually absent

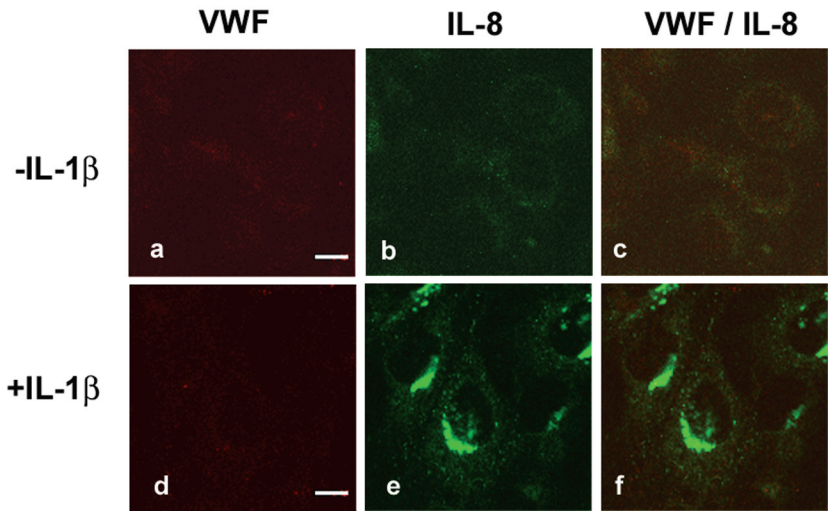

Figure 2. Expression of VWF and IL-8 by EC-RF24 cells. EC-RF24 cells untreated (**a-c**) or treated for 24 h with 10 ng/ml IL-1β (**d-f**). Cells were examined for expression of VWF and IL-8 by double immunofluorescence. VWF staining (in red, **a,d**) was performed with a monoclonal anti-VWF antibody followed by incubation with Texas-red-conjugated goat anti-mouse. IL-8 (in green, **b,e**) was detected using a biotinylated polyclonal anti-IL-8 antibody, followed and FITC-conjugated streptavidin. Merges of the VWF and IL-8 images, **c,f.** Scale bars, 10 μm.

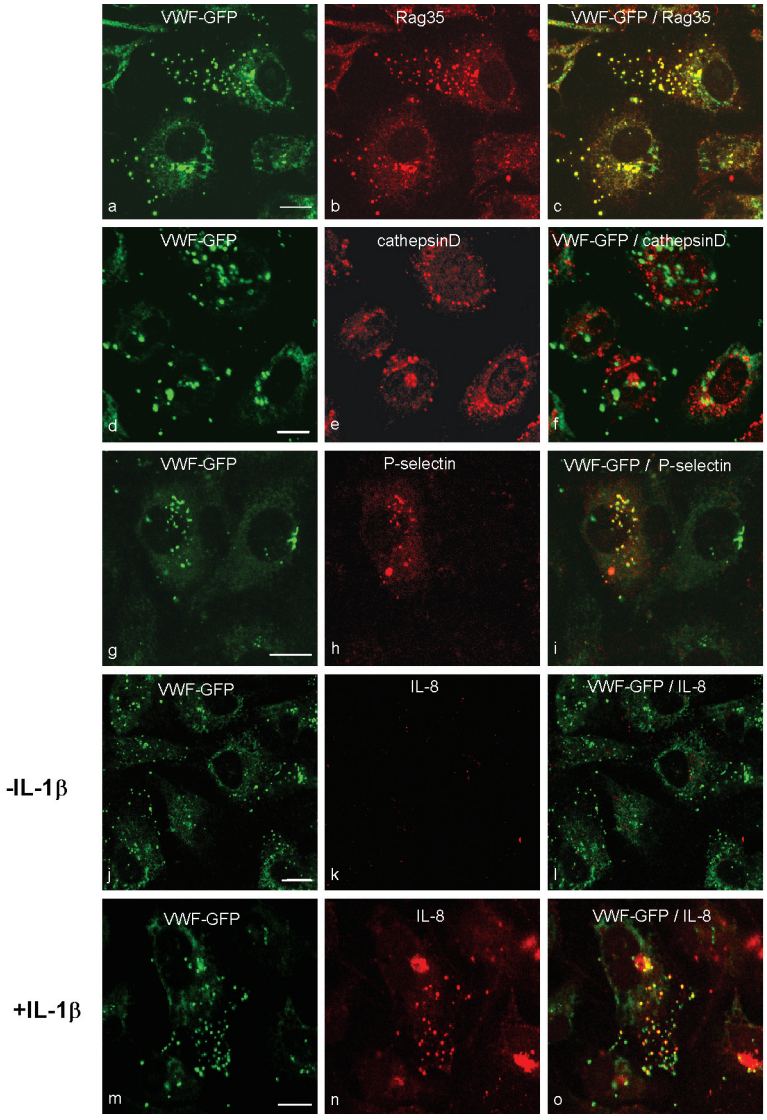

Figure 3. Vesicular localization of VWF-GFP and IL-8 in transduced EC-RF24. Vesicle formation by VWF-GFP-infected EC-RF24 cells was characterized by immunofluorescence. In all panels, VWF-GFP was visualized with the GFP marker (green). Localization of VWF was determined using a monoclonal antibody (CLB-RAg35) directed against the A1 domain of VWF, followed by Texas red labelled goat anti mouse IgG (**b,c**). Localization of cathepsin D was determined with anti-human cathepsin D polyclonal antiserum, followed by Texas-red-conjugated horse-anti-rabbit IgG (**e,f**). Sequential incubation of monoclonal anti-P-selectin antibody RUU 1.18, and Texas Red-labeled goat-anti-mouse IgG was performed for P-selectin detection (**h-i**). To determine the IL-8 localization in IL-1β untreated (**j-l**) or treated (**m-o**) cells, a biotinylated polyclonal sheep anti-human IL-8 antiserum was used, followed by Alexa594-labeled streptavidin incubation (**k,l,n,o**). The antibody against VWF co-localized entirely with GFP (**c**). VWF-GFP did not co-localize with the lysosomal marker cathepsin D (**f**). However, co-localization of VWF-GFP containing vesicles with P-selectin (**i**) and with IL-8 (**o**) (only upon IL-1β stimulation) is shown in yellow. Scale bars, 10 μm.

(Fig.2d) whereas IL-8 expression was only found in structures characteristic of the Golgi apparatus (Fig.2e). As expected, no co-localization of VWF and IL-8 was observed (Fig.2f). These observations suggest that in endothelial cells with reduced VWF-synthesizing capacity, IL-8 targeting into Weibel-Palade bodies is defective.

Co-localization of IL-8 and VWF in VWF-GFP infected EC-RF24 cells.

To further substantiate this hypothesis, VWF synthesis was restored in EC-RF24 cells by retroviral transduction. These cells were used for co-localization studies of de novo synthesized VWF and IL-8. To discriminate between sporadic, endogenous VWF expression and transgenic VWF, EC-RF24 cells were transfected with a VWF-GFP hybrid vector (see Material and Methods). Fluorescence microscopical analysis demonstrated the formation of granular, green fluorescent vesicles in infected EC-RF24 cells (Fig.3a,d,g,j,m). Staining of transfected cells with a monoclonal antibody (CLB-RAg35) directed against the A1 domain of VWF (Fig.3b) showed entire co-localization between GFP and VWF (Fig.3a-c). This observation suggests that the GFP tag is an integral part of the VWF molecule. This is supported by the observation that VWF-GFP secreted by EC-RF24-VWF-GFP showed identical multimeric patterns after staining with either anti-VWF or anti-GFP antibodies (Fig.4).

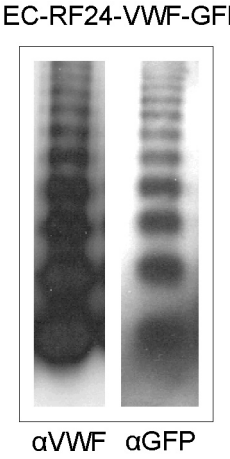

Figure 4. Multimer analysis of VWF-GFP. VWF-GFP-infected EC-RF24 were stimulated for 60 min with 1U/ml thrombin. Released VWF was concentrated by S-Sepharose, run on a 1.4% agarose gel, and blotted. VWF multimers produced by EC-RF24-VWF-GFP react identically to either anti-VWF or anti-GFP antibodies.

The vesicles were distinct from lysosomes as the GFP-associated vesicles did not co-localize with granules that stained with a Texas red-conjugated antibody to cathepsin D, a lysosomal marker (Fig.3e,f). On the other hand, VWF-GFP did co-localize with P-selectin, a typical marker of Weibel-Palade bodies (Fig.3g-i). Like uninfected EC-RF24 cells, VWF-GFP-infected cells did not stain for IL-8 (Fig.3j-l). On the other hand, pre-treatment of the VWF-GFP-infected cells for 24 h with IL-1β resulted in significant co-localization of de novo synthesized IL-8 and VWF-GFP (Fig.3m-o). These observations suggest that in endothelial cells VWF functions as

a helper protein in the sorting of IL-8 and P-selectin to Weibel-Palade bodies.

Visual inspection of live cells stimulated with PMA during 60 min showed that the number of granules decreased under these conditions, suggesting that regulated exocytosis had occurred. Also some non-stimulated control cells displayed loss of granules during 60 min incubation period under the microscope. However, the decrease of granule number was less apparent than the decrease observed in the presence of PMA. To document regulated exocytosis in a more quantitative manner, the number of vesicles was counted at the single cell level in 30-40 cells before and 60 min after stimulation. This analysis revealed that the decrease of granule number in PMA treated cells was significantly larger than the decrease in vesicle number of control cells (Table 1), indicating that indeed the VWF-GFP containing vesicles may undergo regulated secretion. Attempts to quantify the amount of either VWF-GFP or IL-8 released by agonist stimulation of the transfected cells have been hampered by the low number of GFP-transfected cells.

Table 1. Significant decrease of the number of Weibel-Palade bodies after stimulation of VWF-GFP transfected cells with PMA.

Stimulus	Average no. WPb per cell (± SEM)		Number of cells	Percentage of initial no. of WPb (%± SEM)
	0 min	60 min		
Control	17.8 ± 1.0	16.2 ± 1.0*	33	90.9 ± 2.2
PMA	24.8 ± 2.7	19.2 ± 2.1*	38	78.4 ± 2.0**

abbreviations: WPb, Weibel-Palade bodies
*p< 0.001 difference between number of WPb counted at 0 and 60 min
**p< 0.001 difference between PMA and control cells

DISCUSSION

In this report two novel approaches were employed to study the role of VWF in controlling the sorting of IL-8 in endothelial cells. First, we used an endothelial cell line (EC-RF24) that closely mimics the phenotypic features of primary endothelial cells but lacks the capacity to produce VWF and Weibel-Palade bodies in substantial amounts. Therefore, this cell line provides a proper model to study the effect of transgenic VWF expression on the biogenesis of Weibel-Palade bodies and targeting of endogenous proteins in endothelial cells. Accumulated evidence indicates that different cell types may use different sorting mechanisms (reviewed in [20;21]).

Sorting pathways of cell lines of non-endothelial origin, previously used to study VWF-associated regulated secretion, such as the mouse pituitary cell line AtT-20 [22;23] or CV-1 cells (monkey kidney cells) [10] do not necessarily reflect endothelial cell sorting mechanisms. Second, a VWF-GFP hybrid was expressed in these cells to enable direct visualization of vesicle formation and to allow discrimination between residual, endogenous VWF and transgenic, tagged VWF. In terms of cellular localization and multimeric distribution the GFP tag appeared to be an integral part of the VWF-GFP hybrid (Fig. 3a-c, Fig.4). Although this was not the primary aim of the present study, this procedure also allows to study the dynamics of Weibel-Palade bodies in living endothelial cells.

This experimental approach revealed that VWF plays an active role in targeting IL-8 into Weibel-Palade bodies. This conclusion is based on the following observations and considerations. (i). Unlike in wild-type endothelial cells, in EC-RF24 cells IL-8 is primarily observed in structures typical of the Golgi apparatus (Fig.2e,f); IL-8 was not found in Weibel-Palade body like vesicles. This suggests that when VWF expression is reduced, IL-8 storage is reduced or absent. (ii). In VWF-GFP-infected EC-RF24 cells, IL-8 is co-targeted with VWF-GFP into Weibel-Palade body-like granules (Fig.3o). VWF-GFP and the lysosomal marker cathepsin D did not co-localize in these cells (Fig.3f), arguing against the possibility that VWF-GFP (and IL-8) was targeted to lysosomes. (iii) P-selectin co-localized with VWF-GFP in VWF-GFP containing vesicles (Fig.3i). (iv) VWF-GFP containing vesicles are capable of regulated exocytosis (Table 1). As most of the VWF-GFP-positive granules colocalize with IL-8 (Fig.3o), this observation suggest that in infected EC-RF24 cells also IL-8 can be secreted through the regulated pathway. Taken together, these observations indicate that in VWF-GFP-infected EC-RF24 cells VWF-GFP and, thus, IL-8 are co-targeted into Weibel-Palade bodies. Apparently, VWF is able to facilitate sorting of structurally unrelated, though selected subset of proteins into the regulated secretory pathway in endothelial cells. Similarly, VWF may also direct the sorting in endothelial cells of CD63, endothelin and α1,3-fucosyltransferase, also residents of Weibel-Palade bodies.

Little is known about the mechanism by which these proteins are targeted into Weibel-Palade bodies. It is possible that these proteins interact or co-aggregate with VWF multimers in TGN, a process that may further facilitate their retention and condensation within the Weibel-Palade bodies [24;25]. Indeed, VWF may bind a variety of proteins and biological compounds [26]. We speculate that other Weibel-Palade body constituents may specifically interact with VWF preceding maturation of secretory vesicles. Pertinent to this point is our preliminary observation that IL-8 may bind to VWF (results not shown).

A recent study demonstrated that the role of VWF and P-selectin can be

intricately linked [12]. VWF-deficient mice present a significant reduction in leukocyte recruitment in experimental inflammation. In these mice, P-selectin is not properly expressed at the plasma membrane after stimulation of endothelial cells by inflammatory mediators such as IL-1β or tumor necrosis factor α. The present study suggests that VWF deficiency could also lead to an aberrant release of IL-8 which, together with reduced expression of P-selectin, might decrease neutrophil recruitment in early phases of inflammation and sites of endothelial cell perturbation. Similarly, it is tempting to speculate that severe VWF deficiency also affects physiological and pathophysiological processes in which regulated mobilization of other residents of Weibel-Palade bodies is required.

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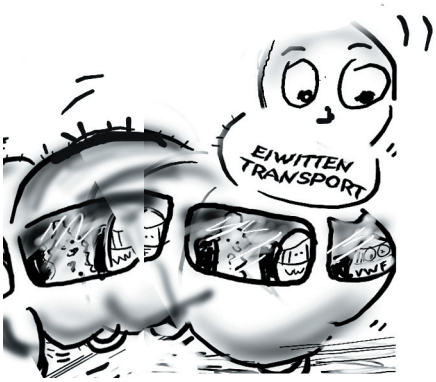
Chapter 3

REAL-TIME IMAGING OF THE DYNAMICS AND SECRETORY BEHAVIOUR OF WEIBEL-PALADE BODIES



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ABSTRACT

Weibel-Palade bodies (WPbs) are specialized secretory granules found in endothelial cells. These vesicles store hormones, enzymes and receptors, and exhibit regulated exocytosis upon cellular stimulation. Here we have directly visualized intracellular trafficking and secretory behavior of WPbs in living cells employing a hybrid protein consisting of von Willebrand factor (VWF), a prominent WPb constituent, and the green fluorescent protein (GFP). Immunofluorescent microscopy demonstrated that this chimera was targeted into WPbs as visualized by its co-localization with endogenous VWF and P-selectin, another WPb resident protein. In unstimulated cells, some WPbs seemed motionless, whereas others moved at low speed (<10 nm/sec) in a stochastic manner. A prominent feature was the formation of membrane-apposed patches upon stimulation of cells with $[Ca^{2+}]_i$- or cAMP-raising secretagogues, suggesting fusion of WPbs with the plasma membrane. Patches remained visible for more than 20 min. This sustained, membrane-associated retention of VWF, or other WPb residents, might play a role in focal adhesion of blood constituents to the endothelium upon vascular injury. In addition, stimulation with cAMP-raising resulted in clustering of a subset of WPbs in the perinuclear region of the cell. Apparently, these WPbs escaped secretion. This feature may provide a mechanism to control regulated exocytosis.

INTRODUCTION

Vascular endothelial cells are equipped with a machinery that, upon perturbation, allows prompt delivery of a number of bioactive substances, including hormones, receptors and adhesive molecules, to the surface of the cell. A distinct subset of proteins destined to be released upon stimulation of the endothelium stems from Weibel-Palade bodies (WPbs), typical and morphologically highly organized storage vesicles that release their contents by regulated exocytosis. WPbs are endothelial cell-specific elongated organelles, enclosed by a limiting membrane, which are about 0.1 μm wide and up to 4 μm long [1] and originate from the trans-Golgi network [2,3]. They serve as storage vesicles for a variety of proteins with different biological functions, such as the leukocyte adhesion receptor P-selectin [4,5], and the chemokine interleukin-8 (IL-8) [6,7]. Effective translocation of P-selectin from WPbs to the cell surface is critical for the binding and rolling of leukocytes on the endothelium at sites of inflammation [8,9]. Similarly, regulated exocytosis of IL-8 provides an effective means for controlling local leukocyte extravasation [10]. One of the most prominent WPb residents is von Willebrand factor (VWF), an adhesive

multimeric glycoprotein that contributes to platelet adhesion and haemostatic plug formation at sites of vascular injury (reviewed in [11,12]. Regulated secretion of VWF provides an adequate means for endothelial cells to actively participate in controlling the arrest of bleeding upon vascular damage. Taken together, it seems likely that regulated exocytosis of WPbs serves several physiological functions including inflammatory and haemostatic responses.

Regulated exocytosis of VWF and other WPb residents involves the translocation of WPbs from the cytoplasm toward the cell surface and the fusion of these vesicles with the plasma membrane. Increased concentrations of cytosolic Ca^{2+} have been implicated in the mechanism of exocytosis of a number of agonists, including thrombin and histamine [13,14]. Release of WPbs can also be induced by secretagogues, such as epinephrine or forskolin, agents known for their ability to activate cAMP-dependent signaling [15,16]. The cellular responses to increased cytosolic Ca^{2+} are most likely mediated by calmodulin and small GTP-binding proteins [17-20]. The molecular mechanisms associated with cAMP-dependent exocytosis of WPbs remain to be identified. Besides the possibility that cAMP triggers a cascade of reactions, including activation of protein kinases, that eventually lead to cellular secretion, little is known about the molecular mechanisms distal to adenylate cyclase activation and cAMP generation. In one respect cAMP-mediated responses differ from regulated secretion elicited by a rise in cytosolic Ca^{2+} in that secretion induced by Ca^{2+}-raising agents involves the release of both peripheral and central granules, whereas cAMP-mediated secretion primarily involves vesicles located in the periphery of the cell [19].

Though studies performed so far have clearly provided the basis for understanding the molecular machinery responsible for exocytotic trafficking of WPbs, little is known about the dynamics of this secretory process. To date, exocytosis of WPbs has been studied only morphologically by monitoring defined stages of this process in fixed cells. These conditions do not necessarily reflect the dynamics found in intact living cells. The aim of this study was to investigate the intracellular trafficking of WPbs in living, wild-type endothelial cells in real time. For this purpose, we introduced, by retroviral transduction, a green fluorescent protein (GFP)-tagged VWF into primary human umbilical vein endothelial cells (HUVEC), a well characterized model of vascular endothelium. The VWF-GFP chimera was correctly processed and targeted to WPbs, together with endogenous VWF and P-selectin. This approach, exploiting the intrinsic fluorescence of VWF-GFP, allows direct visualization of the routing and fate of WPbs upon stimulation of the cell. Our data reveal novel features of the dynamics and secretory behavior of WPbs, including perinuclear redistribution and membrane-apposed accumulation of GFP-containing granules. This study also supports the role of WPb constituents in controlling adher-

ence of circulating blood cells to activated endothelium.

MATERIALS AND METHODS

Cell culture

Freshly isolated HUVEC and VWF-GFP infected HUVEC were cultured in medium containing an equal amount of RPMI 1640 and Medium 199 (Gibco BRL, Paisley, U.K.), supplemented with 10% human serum, 10% fetal calf serum, 2 mM glutamine (Merck, Darmstadt, Germany), 100 units/ml penicillin, 100 μg/ml streptomycin (Gibco), 5 units/ml heparin (Leo Pharmaceutical Products, Weesp, The Netherlands), and 2.5 ng/ml fibroblast growth factor-Basic (Sigma Chemical Co., St Louis, Mo., U.S.A.) [21].

Construction of the LZRS IRES VWF-GFP vector

VWF-GFP was constructed by replacing the VWF A2 domain by the sequence encoding the GFP (Fig.1A). The GFP fragment was amplified from the pEGFP-N3 vector by PCR using primers containing the XmaI and BsPI restriction sites flanking the A2 domain and the ends of the GFP coding sequence. The primers used were as follows: GFP-sense: 5'-AATCCCGGGGGTGAGCAAGGGCGAGGAG-3'; GFP-antisense: 5'-AATTCCGGACTTGTACAGCTCGTCCAT-3'. A recombinant retroviral vector for delivery of the VWF-GFP gene was constructed using the dicistronic retroviral LZRS vector as described previously [22]. The fragment was inserted downstream of the 5' long terminal repeat (LTR) using EcoRI restriction site.

Retroviral gene transduction

Helper-free recombinant retrovirus was produced after transfection of the LZRS-VWF-GFP DNA into Phoenix-A cells (a 293T-based amphotropic retroviral packaging cell line) [23] by calcium phosphate transfection (Gibco). The cells were cultured in medium containing IMDM, 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin (all from Gibco) and 2 μg/ml puromycin (Sigma). For the production of virus, Phoenix-A cells were maintained for at least 6 h in medium lacking puromycin. HUVEC were grown on 35 mm-diameter fibronectin-coated wells until they reached a cell density of 20-40%. Cells were subsequently transduced with the harvested virus supernatant of the Phoenix cells [24]. The transduction efficiency was tested by determining the percentage of GFP positive cells using flow cytometry analysis with a FACSscan (Becton Dickinson, San Jose, CA, USA). HUVEC were also transduced with the VWF-GFP vector together with the human telomerase reverse transcriptase (hTERT) gene. This was performed

by co-transduction of LZRS-VWF-GFP and LZRS-hTERT-NGFR virus (1:1 v/v) (a generous gift from Dr. H. Spits, Dutch Cancer Institute, Amsterdam, The Netherlands). The LZRS-hTERT-NGFR construct was made by replacing GFP present in LZRS-hTERT-GFP [23] by a truncated form of the neural growth factor receptor (NGFR). NGFR is expressed downstream hTERT and was used as a marker for hTERT transfection.

Multimer analysis

HUVEC and VWF-GFP-infected HUVEC were grown in fibronectin coated 80 cm^2 flasks. At confluence, cells were incubated for 60 min in 6 ml serum-free medium containing 1U/ml thrombin (Sigma). Cell supernatants were collected and cleared of cell debris by centrifugation. The medium containing VWF was concentrated by incubation with 0.5 volume of buffer containing 50 mM imidazol pH 6.5, 100 mM NaCl, 10 mM benzamidine and 1% (v/v) S-Sepharose Fast Flow (Pharmacia; Uppsala, Sweden) for 20 h. S-Sepharose was collected and suspended in sample buffer (9.7 M urea, 4 ml 10% SDS-solution, 1 ml 0.5% bromophenol blue solution and 5 ml stacking gel buffer, pH 6.7 containing 70 mM Tris, 4 mM Na_2EDTA and 0.4% SDS) and boiled for 5 min. Samples (5-10 ng) were layered on a 1.4% SeaKem HGT (P) agarose gel (BioWhittaker Molecular Applications; Rockland, ME USA), run for 2 h at 100 V and 45 mA and blotted on a polyvinylidene difluoride filter (Immobilon-P transfer membranes; Millipore Corporations, Bedford). Finally, blots were incubated with anti-GFP (PoAb A11122, Molecular Probes, Eugene, Oregon, U.S.A) and HRP-labeled horse-anti-rabbit antibodies or HRP-labeled anti-VWF (DAKO, Denmark A/S, Glostrup, Denmark) polyclonal antibodies. Multimers were visualized by ECL (Roche Diagnostics Nederland B.V., Almere, The Netherlands).

Immunofluorescence

Cells were grown to confluence on 1 cm-diameter gelatin-coated glass coverslips, fixed in 3.7% (v/v) formaldehyde and permeabilized for 30 min with PBS buffer containing 0.02% saponin (Sigma) and 1% bovine serum albumin. The cells were then incubated with unconjugated primary antibodies in the permeabilizing buffer for 1 h at 37°C, washed three times, and incubated with conjugated secondary antibodies, as indicated in the figure legends. VWF was visualized with a monoclonal antibody directed against either the A1 domain (CLB-RAg 35) [25] or the A2 domain (CLB-RAg 50) [26] of VWF. A rabbit anti-human cathepsin D polyclonal antiserum (a generous gift from Dr. J.M.F.G. Aerts, Department of Biochemistry, Academic Medical Center, Amsterdam, The Netherlands) was used as a lysosomal marker. The monoclonal antibody to P-selectin (RUU 1.18) was kindly provided by Dr H. K. Nieuwenhuis, University Medical Center Utrecht, Utrecht, The Netherlands

[27]. As secondary antibody, we used Texas-Red -conjugated goat-anti-mouse IgG or horse-anti-rabbit IgG (Vector Laboratories, Burlington, CA, USA). Cells were embedded in Vectashield mounting medium (Vector Laboratories) and viewed on a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, Heidelberg, Germany).

Real-time imaging and microscopy

HUVEC-VWF-GFP were grown on 35 mm-diameter gelatin-coated glass coverslips. At confluence, cells were mounted in an incubation chamber (LaCon, Germany) in HEPES medium consisting of 20 mM Hepes, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 1.2 mM K_2HPO_4, supplemented with 1 mg/ml glucose and 2% human serum albumin. Confocal images were recorded with the Zeiss LSM510 using standard settings for GFP and Texas-Red detection. When required, images were recorded in the multi-track mode (sequential scanning) to exclude interchannel crosstalk. During real-time analysis, the sample chamber was maintained at 37°C using a thermostatted chamber holder. Time-lapse images of WPbs in living HUVEC-VWF-GFP were generated by repetitive imaging at the same focal plane or by making series of optical sections (Z-stacks, 400 nm interval/thickness, 12-15 slices/cell) every 2-5 min. Selected cells were first imaged for 10 min in their initial medium. Subsequently, 10 µl of the secretagogue of interest (final concentrations 1 U/ml thrombin, 50 ng/ml PMA, 10 µM forskolin / 100 µM IBMX or 10 µM epinephrine / 100 µM IBMX; all from Sigma) was carefully added to the medium in order not to disturb the cells or change the focal plane. The recording was continued for another 60 or 90 min. Top view of the cells was performed using the *xy* axis. Three-dimensional analysis was performed using depth-coding software that allowed the monitoring of WPbs translocation along the *z*-axis. This software enabled division of cells in three areas (the apical, the central and the basal area) by encoding each part with a color. Lateral analysis of WPbs was visualized by plotting the cells as *yz* diagrams.

VWF secretion

Regulated secretion of VWF-GFP was investigated either by determining the amount of secreted VWF-GFP antigen or by assessing the decrease of the number of WPbs in living cells after stimulation. HUVEC were grown in 35 mm-diameter fibronectin-coated wells and were used at confluence. Cells were washed three times with PBS and were stimulated for 60 min either with serum-free medium alone or with medium containing 50 ng/ml PMA. VWF antigen concentrations (nM) were measured by ELISA as described previously [28]. VWF-GFP levels (expressed as absorbance at 550 nm) were measured by ELISA using a monoclonal antibody

raised against GFP (MoAb 3E6 from Molecular probes) as coating antibody and an HRP-labeled anti-VWF polyclonal antibodies (DAKO) as conjugate. Results are expressed in relative values, i.e. as a percentage of release from unstimulated control cells from the same cell preparation. To measure the decrease of the number of WPbs, real time imaging was performed on living cells (see previous paragraph). Cells were stimulated with different secretagogues (1 U/ml thrombin, 50 ng/ml PMA, or 10 µM forskolin / 100 µM IBMX), Z-stacks were taken before and 60 min after stimulation and the number of WPbs was counted. The number obtained at t=0 was set at 100%.

RESULTS

VWF-GFP expression and distribution in HUVEC

In initial experiments, HUVEC were retrovirally transfected with either the VWF-GFP hybrid protein (Fig. 1A) alone, or, to extend the life span [29], together with hTERT-NGFR. In terms of the expression and localization of VWF-GFP in cells and trafficking of WPbs, we observed no differences between these transfected cells. In this study results shown are both from VWF-GFP/hTERT-NGFR- and VWF-GFP-transfected HUVEC. The respective cell line used is indicated. The transduction efficiency was approximately 20%, as determined by measuring GFP expression by flow cytometry (not shown). Similarly, analysis by confocal microscopy showed that about 20% of the cells were GFP-positive. Positive cells revealed a vesicular distribution of the green fluorescence (Fig. 1B). Three-dimensional analysis of the cells showed rod shaped vesicles that were distributed throughout the cytoplasm. Closer examination of the position of the GFP-containing organelles within the cell, visualized with a color scale (Fig. 1B), revealed that some vesicles were located at the bottom of the cell (red vesicles), whereas the majority was positioned in the middle (green vesicles) and some others were located at the top of the cell (blue vesicles). Thus, transfection of HUVEC with VWF-GFP results in the formation of GFP-labeled vesicles, typical of WPbs, which are heterogeneously distributed throughout the cell.

We next confirmed whether VWF-GFP was indeed targeted to genuine WPbs. Staining of VWF-GFP-infected HUVEC with anti-VWF (CLB-RAg 35) revealed rod-shaped granules that were always co-localized with the GFP signal, consistent with targeting of VWF-GFP to WPbs (Fig. 2a-c). Similarly, VWF-GFP was co-localized with P-selectin, a typical marker of WPbs (Fig. 2g-i). GFP-positive vesicles were distinct from lysosomes, as GFP-associated vesicles were not co-localized with granules that stained with a Texas red-conjugated antibody to cathepsin D, a ly-

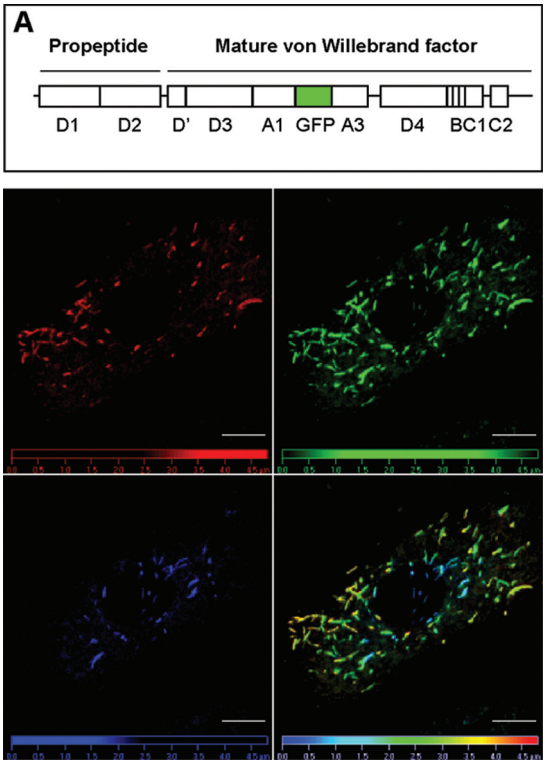

Figure 1. HUVEC transduced with VWF-GFP. (**A**) Schematic representation of the primary structure of the pro-VWF-GFP chimera used for the expression of VWF-GFP. The location of conserved structural domains of the propeptide and mature VWF moiety is indicated. The A2 domain of VWF was replaced by GFP (green) allowing direct visualization of hybrid VWF-GFP. (**B**) Localization of VWF-GFP-containing vesicles in transduced HUVEC. The VWF-GFP vector shown in A was retrovirally transduced in wild-type HUVEC. VWF-GFP was only observed in rod-shaped vesicles. The distribution of VWF-GFP-expressing vesicles in the cell was determined by three-dimensional analysis of the cells using depth-coding software (see Materials and Methods). This software enabled division of cells in three areas (the apical, the central and the basal area) by encoding each part with a color. Red-coded vesicles are located at the bottom of the cell (upper left panel), green in the middle region (upper right panel) and blue at the top of the cell (lower left panel). The merged image of all parts is shown in the lower right panel. Scale bars, 10 μm.

sosomal marker (Fig. 2j-l). These data demonstrate that VWF-GFP is targeted to WPbs together with endogenous, wild-type VWF.

Since the monoclonal antibody CLB-RAg 35 used for VWF staining is directed against a domain (A1 domain) present in both VWF and VWF-GFP, WPbs may contain either a mixture of endogenous VWF and VWF-GFP or only the VWF-GFP chimera. To discriminate between endogenous VWF and VWF-GFP, cells were stained with an anti-VWF antibody (CLB-RAg 50) directed against the A2 domain of VWF. All GFP-positive vesicles stained with this antibody (Fig. 2d-f). This indicates that WPbs of VWF-GFP-infected HUVEC contain both endogenous, wild-type VWF and the VWF-GFP hybrid protein.

VWF-GFP multimerization and secretion

Polymerization of VWF is one of the most characteristic events that occur during its post-translational maturation. Only VWF in its multimeric form is stored in WPbs [11]. To verify whether VWF-GFP is also able to multimerize, wild-type and VWF-GFP-transfected HUVEC were stimulated with thrombin and the respective media were subjected to multimer analysis.

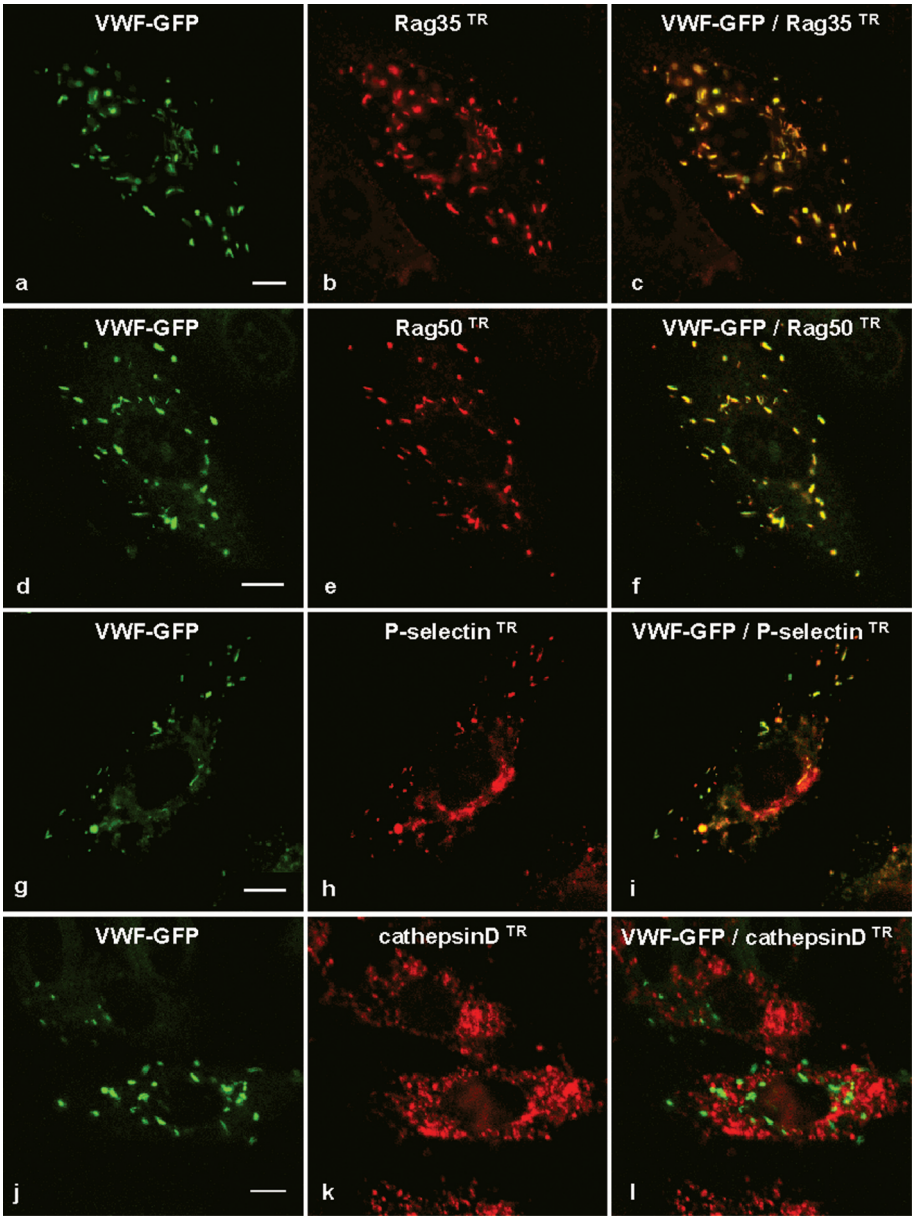

Figure 2. VWF-GFP is co-localized with endogenous VWF and P-selectin in WPbs. VWF-GFP-infected HUVEC were fixed and examined by immunofluorescence microscopy. VWF-GFP-containing vesicles were directly visualized (**a,d,g,j**). Cells were stained either with a monoclonal antibody, CLB-RAg 35, directed against the VWF-A1 domain (**b,c**) or an antibody, CLB-RAg 50, against the VWF-A2 domain (**e,f**), followed by Texas-red-conjugated goat anti-mouse IgG. Monoclonal anti-P-selectin antibody RUU 1.18, and Texas Red-labeled goat-anti-mouse IgG were used for P-selectin detection (**h,i**). Co-localization of VWF-GFP containing vesicles with P-selectin (**i**) is shown in yellow. Localization of cathepsin D was determined with anti-human cathepsin D polyclonal antiserum, followed by Texas-red-conjugated horse-anti-rabbit IgG (**k,l**). VWF-GFP was not co-localized with the lysosomal marker cathepsin D (**l**). Scale bars, 10 μm.

VWF-GFP-transfected cells produced VWF multimers that were similar to wild-type VWF multimers (Fig.3, lane 1 and 2). As expected, VWF multimers produced by wild-type HUVEC did not stain with an antibody against GFP (Fig. 3, lane 3). However, the distribution of multimers secreted by VWF-GFP HUVEC revealed by staining with anti-GFP was identical to the pattern observed after staining with polyclonal antibodies to VWF (Fig. 3, lane 4). Thus, the replacement of the A2 domain by GFP did not affect the ability of VWF to multimerize. Slight differences in mobility most likely reflect differences in size between the A2 domain (191 amino acids) present in wild-type VWF and the inserted GFP (239 amino acids).

Figure 3. Multimer analysis of VWF-GFP. Wild-type HUVEC (lanes 1 and 3) as well as VWF-GFP-infected HUVEC (lanes 2 and 4) were stimulated for 60 min with 1U/ml thrombin. Released VWF was concentrated by S-Sepharose, run on a 1.4% agarose gel, and blotted. Blots were incubated either with anti-VWF (left panel) or with anti-GFP (right panel). VWF multimers produced by HUVEC-VWF-GFP (lane 2) are similar to wild-type multimer forms (lane 1). VWF-GFP multimers also react with the anti-GFP antibody (lane 5), in contrast to wild-type VWF (lane 3).

We also quantified the secreted VWF-GFP. Stimulation with PMA for 60 min resulted in a 1.5 fold increase of VWF ($p<0.05$) and GFP antigen ($p<0.01$) secreted in the medium compared to the amounts secreted by unstimulated cells (Fig. 4). Similarly, after incubation with thrombin, PMA or forskolin for 60 min, we observed a significant decrease of the number of WPbs compared with the number secreted by untreated cells (Table 1). Collectively, these data indicate that WPbs containing VWF-GFP retain their ability to secrete their cargo in a regulated manner.

Dynamics of WPbs in resting VWF-GFP-transfected HUVEC

Having established that VWF-GFP properly accumulated in WPbs, was releasable and had retained its ability to multimerize, we first examined the dynamics of WPbs in living resting cells. To follow individual vesicles we selected cells with relatively few WPbs. These vesicles were monitored for 60 min at intervals of 2 min.

Figure 5 shows individual frames of the first 40 min (at intervals of 10 min) of a real time movie of resting VWF-GFP-transfected cells (Fig5video1.mov). We observed vesicle traffic in an apparently random and uncoordinated fashion through-

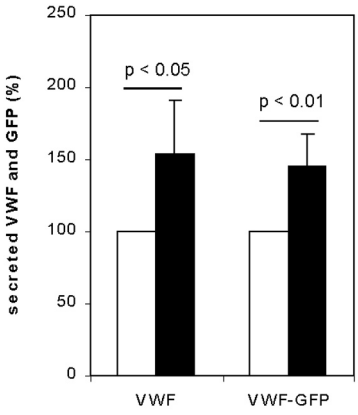

Figure 4. Regulated secretion of VWF and GFP by VWF-GFP-transfected cells. Confluent VWF-GFP-infected HUVEC grown in 35 mm-diameter wells were incubated for 60 min with medium containing 50 ng/ml PMA or with medium alone. Released VWF-GFP was assessed by measuring VWF (nM) and GFP (A550) antigen. Results are expressed in relative values. VWF and GFP release from unstimulated cells was defined as 100%. Results are the mean ± SEM of 3 experiments.

Table 1. Decrease of WPb number from VWF-GFP-infected HUVEC induced by different secretagogues

secretagogue	decrease (% ± SD)	p
control	3.6 ± 9.4	NS
thrombin	24.3 ± 13.3	0.001
PMA	30.4 ± 11.3	0.001
forskolin	40.4 ± 12.3*	< 0.01

Cells were incubated with 1 U/ml thrombin, 50 ng/ml PMA, 10 µM forskolin / 100 µM IBMX or medium alone (control), and monitored in real-time. The number of WPbs was counted in individual cells before and after 60 min of incubation. The relative decrease (in %) of vesicle number after 60 min is depicted in this table. [a] The number of WPbs was difficult to assess due to strong perinuclear clustering (cf. Fig. 9)

out the cell body. Some granules seemed motionless during the entire recording period as if they were tethered (Fig. 5, WPb 1,3), whereas others were continuously moving in a stochastic manner (Fig. 5, WPb 2,4-7). Some vesicles seemed to travel longer distances, notably toward the periphery (Fig. 5, WPb 4,7), whereas others returned to their starting point (Fig. 5, WPb 2,5). We also observed WPbs that rotated along their longitudinal axis and sometimes appeared as round vesicles (Fig. 5, WPb 6). Under these conditions WPbs moved with velocities of up to 10 nm/s. Compared to traffic of secretory granules in other cell types their movement is extremely slow (e.g.[30]).

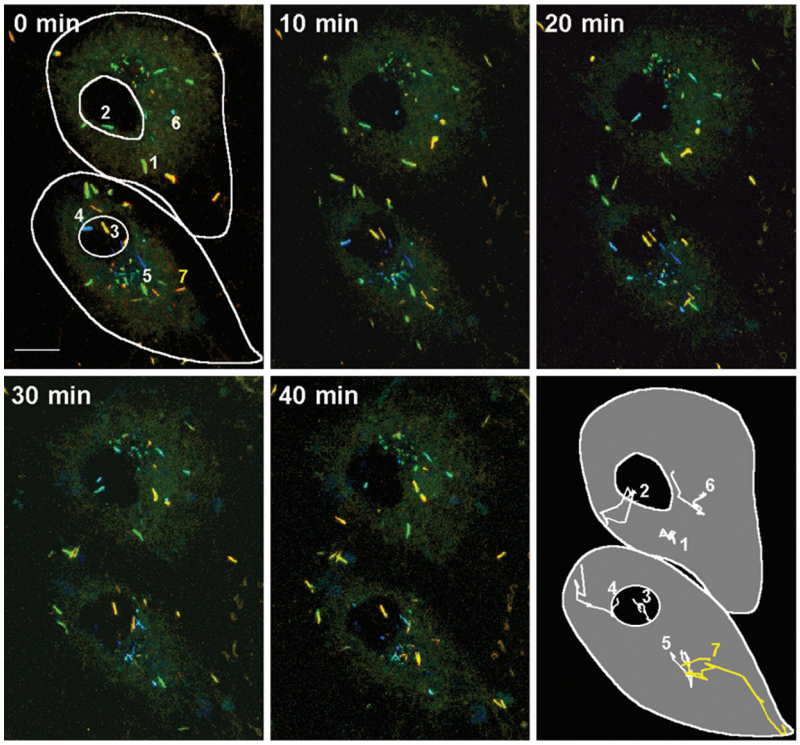

Figure 5. Dynamics of WPbs in resting cells. Living VWF-GFP/hTERT-NGFR transduced HUVEC were monitored in real-time at intervals of 2 min during 54 min of incubation with medium. 5 individual frames (0, 10, 20, 30 and 40 min) of the real-time movie (Fig5video1.mov) are shown. WPbs of interest are numbered on top or at the right side. WPbs showed a variety of motions. WPb 1 and 3 seemed motionless. Other WPbs seem to move in a random fashion. However, some covered long distances toward the periphery (WPb 4,7) whereas others returned to their original position (WPb 2,5). WPb 6 moved perpendicularly to the plasma membrane. Scale bar, 10 μm.

Dynamics of WPbs in PMA-stimulated HUVEC-VWF-GFP

We next investigated WPb trafficking induced by different agonists such as thrombin, PMA, forskolin and epinephrine. Cells were monitored during 60 min of stimulation at intervals of 1 min. Figure 6 shows a typical time-lapse sequence of VWF-GFP-infected HUVEC stimulated with PMA (Fig6video2.mov).

The apparent random movement of WPbs changed radically upon stimulation with PMA. In each real-time stimulation experiment performed (n=8) we observed the same succession of events. Cells contracted slightly which coincided with a slight movement of WPbs toward the center of the cell. Vesicles were not seen to clearly move to the periphery of the cell. However, approximately 15 min after stimulation of the cells, the rod-shaped WPbs transformed into very bright, stationary patches (Fig. 6A, WPb 1,2,3; Fig. 6B). Typically, individual patches remained

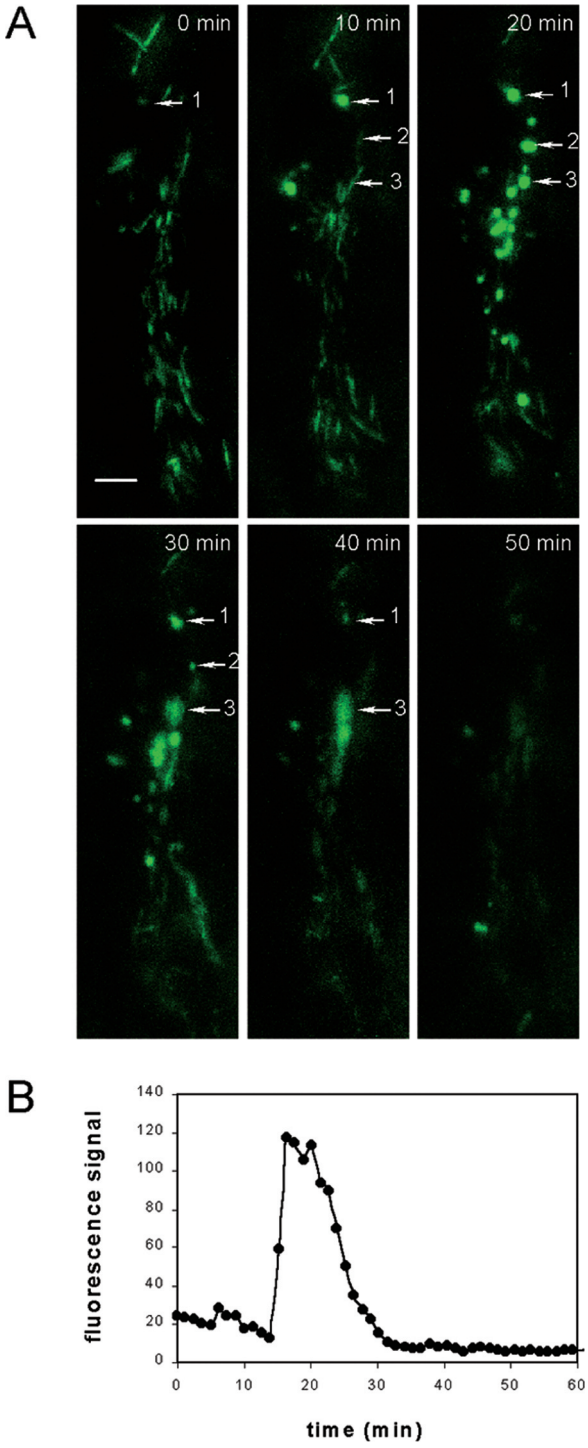

Figure 6. Dynamics of WPbs upon stimulation of HUVEC-VWF-GFP with PMA. Living VWF-GFP/hTERT-NGFR transduced HUVEC were monitored in real-time at intervals of 1 min during 54 min of incubation with 50 ng/ml PMA (see also Fig6video2.mov). **A.** WPb trafficking of one single cell is shown at intervals of 10 min. During the first 10 min WPbs showed the same behavior as in resting cells (Fig. 4). However, after 10 min of incubation with PMA, WPbs started to transform into highly GFP-condensed patches. Patches were not formed simultaneously (WPb 1, 10 min; WPb 2 and 3, 20 min). However, most of the patches were observed 20 min after stimulation with PMA. Patches lasted on average 20 min (WPb 1-3). Some patches formed a cloud before completely disappearing (WPb 1 and 3, 40 min). 60 min after stimulation the cell was devoid of WPbs. Scale bar, 5 μm. **B.** Total fluorescence intensity produced by WPb 2 during the whole recording period. The fluorescence was measured using the LSM510 function "Mean Region of Interest". Note the sharp increase of the fluorescence signal that is produced upon formation of the patch. The gradual fading of the patch correlates with the long trailing edge of the fluorescence peak.

visible for approximately 20 min. Finally, these patches completely disappeared and the cells became depleted of fluorescent material. Interestingly, disappearance of a patch often coincided with the appearance of a diffuse “cloud” (Fig. 6, WPb 2, 30 min and WPb 3, 40 min) at the surface of the cell. This feature most likely reflects gradual dispersion of VWF-GFP into the extracellular environment. The formation of patches of a single cell was not synchronized. The first patches appeared on average 15 min after stimulation; others appeared only after 60 min. Similarly, patches disappeared at apparently different rates. Cells were totally depleted of WPbs between 60 and 120 min after stimulation.

Figure 7 shows a three-dimensional analysis of the transformation of a single WPb into a patch (arrow). To facilitate this analysis, we selected a cell with few vesicles. The *xy* view of this cell (Fig. 7A) shows that during the first 20 min of stimulation with PMA, the WPb appeared as a thin, rod-shaped vesicle. Its depth-coding staining signal suggested that the vesicle was located in the middle of the cell (green color). Indeed, a *yz* view of this WPb (Fig. 7B) confirmed its tubular morphology and central localization. About 20 min after stimulation, this vesicle transformed into a bright round patch located at the apical side (blue depth coding Fig. 7A, 20 min). Examination of the cell on the *xy* plane indicated that the patch indeed represented the same vesicle (Fig. 7B, arrow). However, it had rotated about 45° and was positioned perpendicularly to the focal plane. The vesicle was apposed to the cell membrane at the apical part of the cell (Fig. 7B). The patch remained visible in the xy view for approximately 20 min (Fig. 7A, 50 min). *YZ* view of the vesicle showed that its size diminished in time (Fig. 7B, 22-40 min). Finally, 50 min after stimulation the vesicle had completely disappeared. It is worth noting that upon stimulation WPbs moved at a similar speed as in untreated cells (<10 nm/s). The entire process of patch formation, disappearance of vesicles and the appearance of VWF-GFP “clouds” (see Fig. 6) upon stimulation with PMA most likely reflects fusion of WPbs with the plasma membrane and secretion of VWF-GFP at the fusion site into the extracellular milieu. These processes were observed at both sides of the cell. However, vesicles that were primarily located at the bottom of the cell tended to form patches at the basal side of the cell, whereas vesicles residing at the upper part of the cell tended to dock at the apical side. We also noted that WPbs localized around the nucleus were often positioned in the upper part of the cell (Fig. 7B). We also observed that patches formed by the majority of WPbs located at the periphery of the cell became localized at the basal part of the cells. Furthermore, patches or “clouds” at the apical side of the cell tended to disappear faster than those at the basal side.

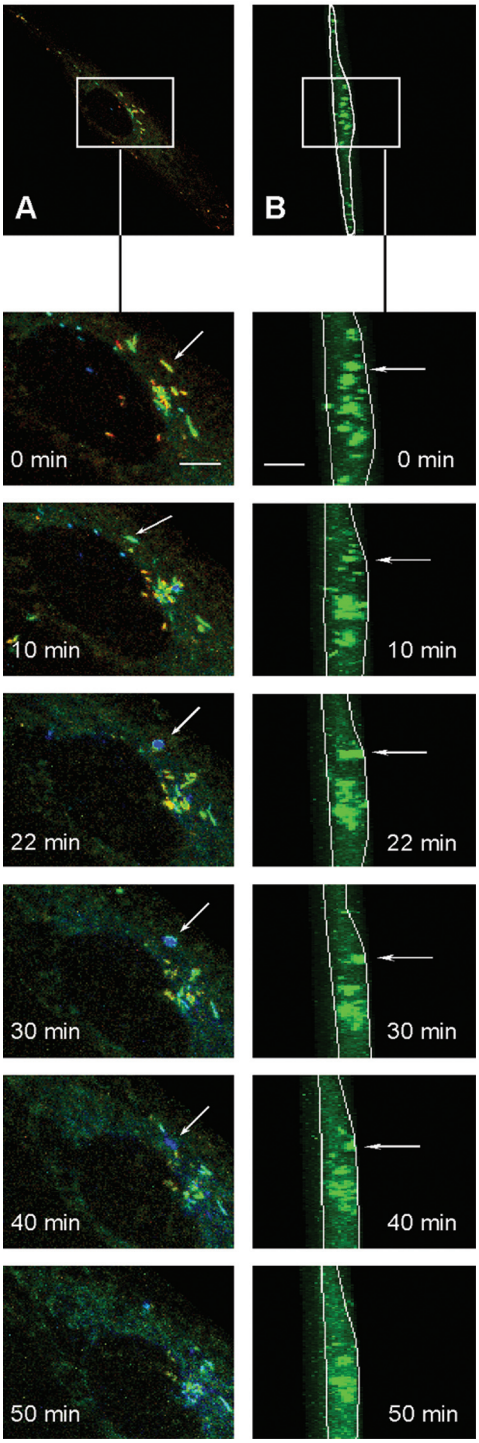

Figure 7. *XY* and *YZ* analysis of a GFP-condensed patch formation. Time-lapsed images of a PMA-stimulated cell showing the transformation of a single WPb into a highly condensed patch. **A.** *XY* view of the cell. About 20 min after stimulation with PMA, the rod-shaped vesicle transformed into a round patch (arrow). The patch phase lasted for approximately 20 min before disappearing (arrow, 22-40 min). The depth of the WPb was coded using the color scale described in Fig.1. Scale bar, 5 μm. **B.** View of the GFP-condensed patch on the *yz* plane. During the first 10 min of stimulation, the *yz* view of the WPb revealed a round conformation (arrow, 0-10 min). After about 20 min, the vesicle was located perpendicularly and apposed to the luminal cell membrane. In time the size of the WPb diminished (arrow, 30-40 min), until it finally disappeared (50 min). Scale bar, 5 μm.

Dynamics of WPbs in thrombin-stimulated HUVEC-VWF-GFP

When VWF-GFP-infected HUVEC were stimulated with thrombin and examined in real time, we often observed an immediate contraction of the cells. This somewhat obscured the visualization of the dynamics of WPb secretion. This process started only a few min after the addition of the stimulus. Thrombin also induced more rapid formation of patches than PMA. Most patches were formed within the first 5 min of stimulation (Fig. 8, 4 min), and they disappeared faster (about 10 min) than PMA-induced patches (Fig. 8, 16 min). In some cases, concomitant formation of clouds was observed (Fig. 8, WPb 2,3, 16 min). It is worth mentioning that fewer patches were formed with thrombin than with PMA. As during PMA- induced stimulation, patch formation and fading were observed at the apical as well as the basal part of the cells. Thus, tentative fusion of WPbs with the plasma membrane induced by thrombin was faster than upon stimulation with PMA.

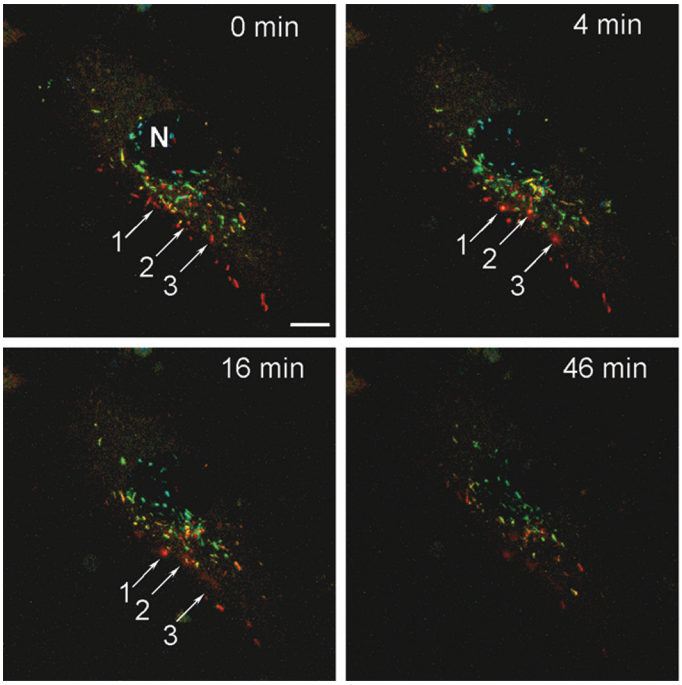

Figure 8. Rapid patch formation induced by thrombin. A VWF-GFP/hTERT-NGFR-transduced cell was stimulated with 1 U/ml thrombin for 60 min. Time-lapse images were taken at intervals of 2 min. The depicted time points (0, 4,16 and 46 min) show the appearance of patches very soon after stimulation (WPb 1-3, 4 min). After 10 min of stimulation, disappearance of the patches coincided with the appearance of clouds (WPb 2 and 3, 16 min). **N**, Nucleus. Scale bar, 10 μm.

WPb dynamics induced by cAMP-agonists

Unlike PMA and thrombin, neither forskolin (not shown) nor epinephrine (Fig. 9) induced contraction of the cell. However, we observed that these secretagogues induced a vectorial migration of WPbs toward the nucleus (Fig. 9). Perinuclear redistribution was much more pronounced than that induced by either PMA or thrombin. After 30 min most of the vesicles clustered around the nucleus in a starlike structure (Figs. 9A, 30-60 min). YZ analysis of the cell demonstrated that the initial random distribution of WPbs changed completely upon stimulation with epinephrine. We clearly observed that upon stimulation, vesicles moved to the center of the cell, in particular to the luminal side (Fig. 9B). Furthermore, vesicles moved up to ten times faster (<100 nm/s) than in resting cells.

During migration, secretion was suggested to occur by means of the formation of patches that started to appear within approximately 20 min of stimulation. However, they disappeared faster than upon PMA stimulation. Indeed, patches lasted for about 5 min (Fig9video3.mov). Patches were seen both at the periphery of the cell and around the nucleus. Furthermore, patch formation was observed at the basal (Fig. 9, WPb 1) as well as the apical (WPb 2) part of the cell. Thus, cAMP agonists induced distinct migration of WPbs to a specific site directly above the nucleus and concomitant formation of a starlike clustering of vesicles. Tentative fusion events started within the same time interval as observed upon stimulation with PMA, but the event itself was as fast as after stimulation with thrombin.

DISCUSSION

Biogenesis and regulated exocytosis of WPbs have been extensively studied by biochemical and morphological analyses of endothelial cells. So far, the dynamics of this unique endothelial-cell specific storage device could not be addressed. In this study the first evidence is presented for the complexity of the dynamics of WPbs. We constructed a VWF-GFP chimera that was properly targeted to WPbs and secreted upon stimulation. This allowed direct visualization of WPb trafficking in living endothelial cells. Our conclusion that VWF-GFP could serve as a genuine WPb marker was based on the following observations: (i) VWF-GFP and endogenous VWF were packaged in the same WPbs (Fig. 2a-f), (ii) VWF-GFP co-localized with a typical WPb resident, P-selectin (Fig. 2g-i), (iii) VWF-GFP was able to multimerize, a typical feature of WPb VWF. The extent of polymerization did not differ from that of wild-type VWF (Fig. 3), and (iv) the VWF-GFP containing WPbs were able to secrete VWF-GFP in a regulated manner (Fig.4, Table 1). Thus, this VWF-GFP species expressed in primary HUVEC behaved, in terms of post-translational

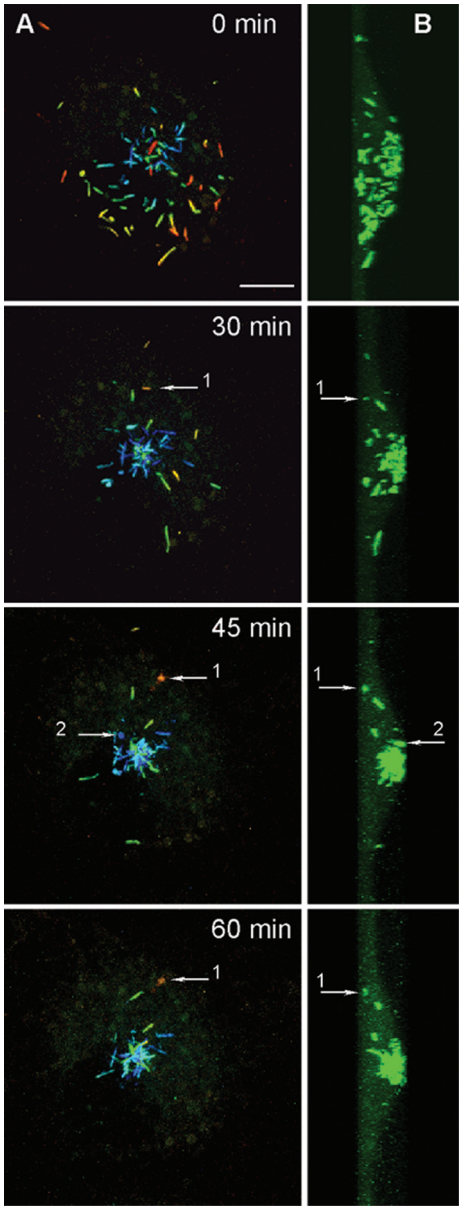

Figure 9. Perinuclear clustering of WPbs induced by epinephrine. A VWF-GFP/hTERT-NGFR-transduced cell that was stimulated with 10 μM epinephrine / 100 μM IBMX for 60 min (see also Fig9video3). Time-lapse images were taken at intervals of 5 min. (**A**) *xy* and (**B**) *yz* views of the cell during stimulation with epinephrine are depicted. Remarkable is the perinuclear recruitment of WPbs that is observed about 30 min after stimulation (A and B, 30-60 min). They seem to be docked at a distinct site proximal to the nucleus, forming a starlike structure (A, 30-60 min). The *yz* view revealed a clustering of the vesicles at the apical side of the cell. Formation of patches was also observed in the *xy* view of the cell after about 45 min of stimulation (A, arrows, 45 min). Examination of the cell on the *yz* plane showed the perpendicular state of the WPbs (B, WPb 2, 45 min). Patches disappeared within 5 min of stimulation (A, WPb 2) or formed a cloud (A, WPb 1, 60 min). Scale bars, 10 μm.

modification and targeting into the regulated secretory pathway, as wild-type VWF and, as such, is a faithful tag of WPb traffic under various experimental conditions.

By monitoring WPb dynamics in live cells we observed that in resting cells these vesicles are not static but consist of pools with different motilities (Fig. 5). Some vesicles barely move as if they were tethered, others seem to travel in a stochastic manner and frequently reverse their direction. Furthermore, all WPbs

moved with remarkably low speed, not exceeding 10 nm/s. Previous studies on other cell types have shown that secretory vesicles may travel in different directions and move with different speed. Newly formed, microtubule-associated vesicles travel at high speed (about 1 μm/s) from the Golgi to the plasma membrane [31-34]. Upon arrival at the plasma membrane, these vesicles are trapped and mature in the dense meshwork of the actin cortex which restricts their motility (about 50 nm/s) [34-36]. Based on these data and on the similar dynamics observed of WPbs, we speculate that tethered WPbs seen in our study (e.g. Fig. 5, WPb 1,3) are docked at the plasma membrane or trapped in the actin cortex of endothelial cells. On the other hand, WPbs that moved in all directions are reminiscent of microtubule-associated granules [31]. It is possible that the lower speed of WPbs is related to their relatively large size compared to that of other secretory granules [32,33]. Whether WPbs examined here are immature or mature granules cannot be concluded from this study. Future studies, using maturation markers and drugs that affect the organization of the cytoskeleton, should define their state of maturation and the role of specific cytoskeletal elements in mediating WPb traffic in more detail.

Stimulation with secretagogues dramatically changes the dynamics of WPbs. We observed two characteristic features of traffic: i) formation of patches observed upon stimulation with both Ca^{2+} and cAMP agonists ii) perinuclear clustering of WPbs only induced by cAMP secretagogues.

Formation of membrane-associated patches

Previously, in several studies with fixed endothelial cells, the formation of large extracellular patches of VWF was noticed upon stimulation of the cells [37-39]. In our study this typical feature is more clearly documented. This is most apparent in the real-time movie of PMA-stimulated cells (Fig6video2.mov). These observations suggest that patch formation reflects fusion of a single WPb with the plasma membrane rather than the clustering of vesicles and subsequent bulk extrusion [38]. We were also able to monitor the gradual release and diffusion of VWF-GFP into the extracellular milieu (Fig. 6,7). This process is reminiscent of fusion events in other cell types monitored by real-time [33,35,40]. Striking was the rather long life span of WPb patches [33]. The rapid (sec) increase of the fluorescence signal due to fusion with the plasma membrane was followed by a slow (min) decay of the fluorescence (Fig. 6B) as VWF-GFP diffused into the extracellular space. The apparent slow release of VWF-GFP from the cell could be due to the compact, crystalloid structure of VWF, which may hamper its dissolution. The possible interaction of VWF with other WPb constituents or with proteins localized at the plasma membrane may also affect the rate of VWF dispersion.

These long lasting patches could have a physiological significance. Patches

at the cell surface may not only reflect diffusion of VWF-GFP but may also provide focal sites with a high concentration of VWF. These sites could play a role in adequately recruiting and binding plasma proteins, blood cells or matrix components to sites of vascular injury. Pertinent to this point is the observation of André and co-workers [41] who showed that platelets adhered rapidly, though transiently, to VWF secreted at the luminal face of endothelial cells upon triggering of the cell with Ca^{2+}-agonists. The observation that adherence of platelets to the endothelium was transient (min) corresponds with the time course of the fading of patches. Although this has not been demonstrated, it is possible that VWF-containing patches also present IL-8, P-selectin or other WPb residents at high concentrations at the cell surface. Focal sites expressing these proteins at high levels could contribute to the rapid recruitment of leukocytes to endothelial cells after simulation. Indeed, the time frame of P-selectin-mediated rolling of leukocytes on the endothelium is in the same order of magnitude as patch fading [42]. It would be fascinating to determine in real-time studies whether indeed blood components interact with WPb constituents at these specific focal sites.

The vectorial movement of WPbs induced by stimulation with either Ca^{2+}- or cAMP raising agents was less prominent than expected on the basis of previous observations. Basolateral as well apical secretion of VWF after simulation of endothelial cells with different secretagogues has been observed [28,43,44]. In this study we observed that WPbs that are recruited toward the nucleus tended to secrete their content at the apical side of the cell whereas vesicles that reside at the periphery of the cell secreted at the basal side. The location of the microtubule-organizing center (MTOC, see below) at the apical side of the cell, might contribute to preferential luminal secretion associated with exocytosis of vesicles located in the vicinity of the nucleus. However, we did not observe any preferential accumulation of WPbs, or a distinct vectorial movement. Quantitative analysis of the dynamics of WPbs in real-time of multiple cells might provide more insight into a possible vectorial behavior of vesicle trafficking.

Perinuclear clustering of WPbs

Another prominent feature of the dynamics of WPbs was the perinuclear clustering of WPbs. Only when cells were exposed to cAMP-raising agonists, such as forskolin or epinephrine, were WPbs docked at a distinct site proximal to the nucleus and spatially organized in a starlike structure (Fig. 9). WPbs migrated to that site at velocities approximately 10 times higher than in resting cells. Based on the observed localization and the morphology of these clusters we assume that the site toward which WPbs migrate is associated with the MTOC, a cytoskeletal element typically located at one side of the nucleus. This observation suggests that micro-

tubuli play a role in the cAMP-induced migration of WPbs. Although not studied here, the cAMP-dependent mechanism responsible for the perinuclear recruitment of WPbs is most likely caused by PKA-dependent modulation of the activity of microtubule-associated motor proteins [45-47]. Further studies should reveal whether MTOC and WPbs are indeed co-localized, and whether cytoskeletal motor proteins are involved in WPb dynamics under these conditions. In addition to perinuclear clustering, also patch formation was observed (Fig. 9, WPb 1,2). Notably, tethered WPbs escaped clustering and fused directly with the plasma membrane. Upon stimulation with thrombin or PMA, WPbs did not accumulate around the nucleus (Fig. 6,8). Apparently, WPbs are directly translocated to the plasma membrane under these conditions. Both actin filaments and microtubules are most likely involved in the Ca^{2+}-dependent trafficking of WPbs [19,48-51].

Taken together, though both cAMP- and Ca^{2+}-raising agents induce patch formation, WPb dynamics triggered by these agonists are clearly different. cAMP-dependent perinuclear recruitment of WPbs may provide a means to limit excessive release of pro-thrombotic and pro-inflammatory mediators stored in WPbs under physiological conditions that raise intracellular levels of cAMP, such as physical exercise or other stress situations [52]. On the other hand, Ca^{2+}-mediated secretion, e.g. in response to vascular damage, most likely reflects mobilization of the entire WPb population to accomplish adequate release of bioactive molecules at sites of vascular injury.

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Chapter 4

SMALL GTP BINDING PROTEIN RAL MODULATES REGULATED EXOCYTOSIS OF VON WILLEBRAND FACTOR BY ENDOTHELIAL CELLS

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ABSTRACT

Weibel-Palade bodies are endothelial cell-specific organelles, which contain von Willebrand factor, P-selectin and several other proteins. Recently, we found that the small GTP-binding protein Ral is present in a subcellular fraction containing Weibel-Palade bodies. In the present study, we investigated whether Ral is involved in regulated exocytosis of Weibel-Palade bodies. Activation of endothelial cells by thrombin resulted in transient cycling of Ral from its inactive GDP- to its active GTP-bound state which coincided with release of VWF. Ral activation and exocytosis of Weibel-Palade bodies were inhibited by incubation with trifluoperazine, an inhibitor of calmodulin, prior to thrombin stimulation. Functional involvement of Ral in exocytosis was further investigated by expression of constitutively active and dominant negative Ral variants in primary endothelial cells. Introduction of active Ral G23V resulted in disappearance of Weibel-Palade bodies from endothelial cells. In contrast, expression of the dominant negative Ral S28N did not affect the amount of Weibel-Palade bodies in transfected cells. These results indicate that Ral is involved in regulated exocytosis of Weibel-Palade bodies by endothelial cells.

INTRODUCTION

Von Willebrand factor (VWF) is a multimeric glycoprotein involved in adhesion of platelets to a damaged vessel wall [1,2]. Synthesis of VWF is confined to endothelial cells and megakaryocytes. During its biosynthesis in endothelial cells, part of VWF is segregated from the bulk flow of proteins and stored in rod-shaped organelles, the Weibel-Palade bodies [3,4]. A number of other components have been identified in Weibel-Palade bodies which include P-selectin, CD63, endothelin and interleukin-8 [5-9]. Upon stimulation with agonists such as thrombin and histamine, Weibel-Palade bodies release their contents into the blood [10-12]. The mechanism of thrombin-induced exocytosis of Weibel-Palade bodies has only been partially elucidated [13-15]. Thrombin induces elevation of intracellular Ca^{2+}-levels which appears crucial for release of VWF from Weibel-Palade bodies [15,16]. Inhibition studies have shown that intracellular Ca^{2+} exerts its effect on regulated secretion of VWF via calmodulin [13,15].

Little attention has been directed at involvement of small GTPases in regulated secretion in endothelial cells. Small GTPases cycle between an active GTP-bound and inactive GDP-bound form. Guanine nucleotide exchange factors (GEFs) enhance the conversion from the inactive GDP-bound to the active GTP-bound form whereas GTPase activating proteins (GAPs) promote GTP-hydrolysis of small GT-

Pases. In many cells, small GTP-binding proteins have been implicated in regulated exocytosis as exemplified by the pivotal role of Rab3A in release of synaptic vesicles at the nerve terminal [17]. Therefore, it seems likely that small GTP-binding proteins are also involved in release of VWF through the regulated pathway in endothelial cells. Recently, we identified the small GTP-binding protein Ral in a subcellular fraction containing Weibel-Palade bodies suggesting a role for this GTPase in regulated exocytosis of these organelles [18]. Ral is a geranylgeranylated GTPase that is ubiquitously expressed [19]. Activated GTP-bound Ral binds to RLIP76 (or Ral binding protein (RalBP)) an effector molecule that possesses GTPase activity for cdc42 and Rac suggesting a link between activation of Ral and rearrangement of the cytoskeleton [20]. Morphological studies have identified Ral on dense granules in platelets and on synaptic vesicles in nerve terminals suggesting a role for Ral in regulated exocytosis [21,22]. Interestingly, Ral has been proposed to interact with calmodulin in Ca^{2+}-dependent manner [23]. Binding to calmodulin enhances GTP-binding to Ral 2-3 fold [24]. These observations may suggest a regulatory role for Ral in calmodulin-mediated release of VWF from endothelial cells.

In this study, we investigated the involvement of Ral in the secretion of Weibel-Palade bodies by endothelial cells. We show that activation of Ral correlates with thrombin induced secretion of VWF from Weibel-Palade bodies. Expression of constitutively active Ral in endothelial cells results in exocytosis of Weibel-Palade bodies whereas expression of a dominant negative Ral variant did not show this effect. Together, these findings suggest that Ral is involved in regulated exocytosis of Weibel-Palade bodies from endothelial cells.

METHODS

Materials

Culture media, trypsin, penicillin, streptomycin and fungizone were from Gibco BRL (Rockville, MD). Human serum was from healthy donors. Heparin (5000 IE/ml) was purchased from Leo Pharmaceutical Products (Weesp, The Netherlands). Bovine fibroblast growth factor and soybean trypsin inhibitor were from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). Chemiluminescence blotting substrate was from Boehringer Mannheim (Mannheim, Germany). Monoclonal antibody CLB-RAg 35 directed against VWF has been described previously [25]. Monoclonal antibody CLB-HEC 75 directed against CD31 has been characterized previously [26]. Peroxidase-conjugated polyclonal rabbit IgG against human VWF was obtained from Dakopatts a/s (Glostrup, Denmark). Monoclonal anti-Ral antibody was from Transduction Laboratories (Lexington, KY). Hybridoma cell line 9E10 was

from ATCC (USA). Protease inhibitors cocktail, Complete™ mini, was from Boehringer Mannheim (Mannheim, Germany). Vectashield was from Vecta Laboratories (Burlington, CA, USA). All chemicals used were of analytical grade.

Cell culture

Endothelial cells were isolated from human umbilical veins and cultured as described previously [18]. Stimulation of endothelial cells by thrombin, Ca^{2+}-ionophore A23187 and phorbol myristic acetate (PMA) was performed as follows. Endothelial cells were washed three times with PBS and cultured for 2 h in M199 medium supplemented with 1% human serum albumin. At the onset of stimulation, the culture medium was replaced by medium containing thrombin, Ca^{2+}-ionophore, PMA or no agonist. To study the effect of the calmodulin inhibitor trifluoperazine (TFP) on thrombin induced secretion, cells were precincubated for 30 min with 40 mM TFP prior to stimulation by thrombin. The amount of VWF secreted from both stimulated and non-stimulated cells was determined in triplicate for each individual time-point.

Ral activation assay

The GTP-bound form of Ral was isolated from total cell lysates by incubating the cell lysate with GST-RalBD coupled to glutathione Sepharose essentially as described previously [27]. Vector pGEX4T3-GST-RalBD was kindly provided by dr. J.L. Bos (Utrecht University, The Netherlands). GST-RalBD was purified from IPTG-induced bacteria as described previously [27]. HUVEC were cultured in 6 wells dishes and grown to confluency. Stimulation of endothelial cells was performed as described in the previous paragraph. At indicated time periods HUVEC were lysed in Ral buffer (15% (v/v) glycerol, 1% NP-40, 50 mM Tris (pH 7.4), 200 mM NaCl, 2.5 mM $MgCl_2$, 1 mM PMSF and 0.1 mM Trasylol). Cell lysates were incubated with 15 mg GST-RalBD precoupled to glutathione Sepharose for 60 min at 4 °C. Beads were washed and analyzed by 12.5 % SDS-PAGE and Western blotting with a monoclonal anti-Ral antibody. All Ral-BD experiments were performed at least three times and yielded similar findings.

Transient expression of Ral and Rab3B variants in HUVEC

Total cDNA of HUVEC and plasmid pGEM-T-Myc-Rab30 were used to construct epitope tagged human Ral, Ral G23V, Ral S28N, Rab3B, Rab3B T36N and Rab3B Q81L [28].

HUVEC were transfected by electroporation using a Genepulser equiped with a RF module (Biorad, Veenendaal, The Netherlands). Confluent HUVEC were trypsinized and 2 million cells were resuspended in 350 ml HEPES-buffered media. Five mg CsCl-purified plasmid was added to the cell suspension and incubated for 5

min at room temperature. Electroporation was performed in 2 mm cuvets at 240 V. Following transfection, cells were seeded on coverslips and cultured for 48 h. Cells were fixed with 3.7 % formaldehyde for 10 min and permeabilized with 0.02% saponin in PBS supplemented with 1% BSA. Cells were then stained with monoclonal anti-myc antibody 9E10 and polyclonal anti-VWF antibody in PBS/0.02% saponin/ 1% BSA. Secondary antibodies used were FITC-labelled goat anti-mouse (CLB, Amsterdam, The Netherlands) and Texas Red-labelled horse anti-rabbit antibodies (Vector Laboratories, Burlinghame, CA). FITC-conjugated CLB-HEC/75 (CLB, Amsterdam, The Netherlands) was used for staining of CD31 in endothelial cells. Cells were embedded in Vectashield mounting medium and viewed by confocal microscopy using a Leica TCS NT (Leica Microsystems, Heidelberg, Germany). Results of two independent experiments are given. The number of Weibel-Palade bodies present in endothelial cells expressing Ral wild type, G23V or S28N was determined. For each construct 20-30 individual transfected cells were evaluated.

RESULTS

Thrombin-induced secretion of von Willebrand factor coincides with activation of Ral

Stimulation of endothelial cells by agents such as thrombin results in exocytosis of Weibel-Palade bodies [10,11]. Recently, we have shown the presence of Ral in a subcellular fraction containing Weibel-Palade bodies [18]. To investigate whether secretion of Weibel-Palade bodies coincides with activation of Ral, HUVEC were stimulated with thrombin for various periods of time (Fig. 1). Exocytosis of Weibel-Palade bodies was determined by measuring release of VWF in the medium. Secretion of VWF initiated 30 sec after the addition of 1 U/ml of thrombin and gradually increased in time (Fig. 1A). The specificity of the Ral binding domain (Ral-BD) of the Ral effector molecule RLIP76 for GTP bound Ral has previously been used to monitor activation of Ral in thrombin stimulated platelets [27]. Similarly, we used GST-tagged Ral-BD to measure activation of Ral in thrombin stimulated endothelial cells (Fig. 1B). A transient activation of Ral was observed which reached a maximum after 2 min of stimulation with thrombin. After 10 min the amount of GTP-bound Ral had decreased significantly. No increase in activation of Ral could be detected in unstimulated cells and the total amount of Ral was similar in all samples analyzed (Fig. 1C). These results show that activation of Ral coincides with release of VWF in endothelial cells following stimulation by thrombin. Similar to thrombin, stimulation of endothelial cells by Ca^{2+}-ionophore A23187 and the phorbol ester PMA also resulted in activation of Ral (please see http://atvb.ahajournals.org).

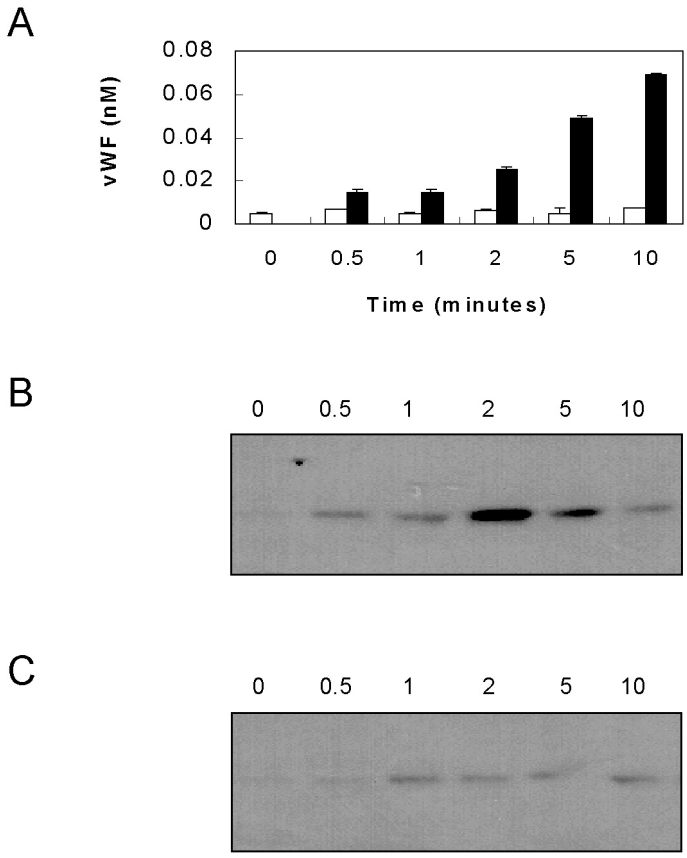

Figure 1. Thrombin-induced secretion of vWF coincides with Ral activation in endothelial cells. Endothelial cells were cultured in 6 wells plates and grown till confluency. Two hours prior to stimulation, culture medium was replaced by M199/1 % (v/v) HSA. Subsequently, cells were stimulated with thrombin (1 U/ml) for the indicated periods of time. Medium was collected and cells were lysed in Ral binding buffer. The concentration of vWF in culture medium was determined by ELISA. Activation of Ral was measured as described in Materials and Methods. (**A**) Concentration of vWF in medium (white bar, control; black bar, stimulated with thrombin); (**B**) Activation of Ral in endothelial cells stimulated with thrombin; (**C**) Activation of Ral in non-stimulated endothelial cells.

Role of calmodulin in activation of Ral and regulated secretion of VWF.

In endothelial cells, calmodulin has been implicated in thrombin-induced exocytosis of Weibel-Palade bodies [12,15]. Recently, a binding site for calmodulin on Ral has been detected and calmodulin has been shown to enhance the binding of GTP to Ral [23,24]. We investigated whether thrombin-induced activation of Ral is affected by antagonists of calmodulin. Endothelial cells were stimulated with thrombin in the presence and absence of calmodulin inhibitor TFP. Two min after the addition

of thrombin, TFP inhibited secretion of VWF by 70%. A slightly lower inhibition was observed at 5 and 10 min following incubation with TFP (Fig. 2A).
In the same series of experiments the effect of TFP on activation of Ral was determined. In the absence of TFP, the amount of GTP-bound Ral increased 6-7 fold following incubation with thrombin for 2 minutes (Fig. 2B and 2C). Incubation with TFP resulted in a 3-fold increase in amount of intracellular GTP-bound Ral at the same time-point (Fig. 2C). At 5 and 10 min following stimulation with thrombin no significant effect of TFP on the activation of Ral was observed (Fig. 2C). Our findings suggest that TFP partially inhibits activation of Ral and release of VWF in endothelial cells stimulated with thrombin.

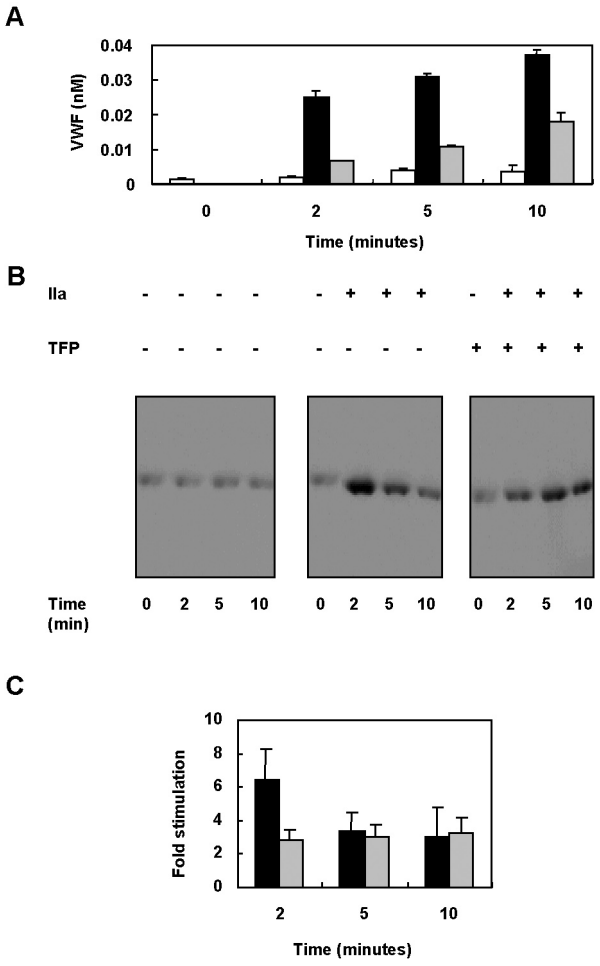

Figure 2. Effect of the calmodulin inhibitor TFP on thrombin-induced secretion of Weibel-Palade bodies and activation of Ral. Endothelial cells were cultured in 6 wells plates and grown till confluency. Two hours prior to stimulation, culture medium was replaced by M199/1 % HSA. Then medium was replaced and TFP was added to a final concentration of 50 mM. In control cells no TFP was added. After 30 min, cells were stimulated with thrombin (IIa; 1 U/ml) for indicated periods of time in the presence/absence of TFP and analysed as described in Materials and Methods. (**A**) Concentration of vWF in culture medium: non stimulated endothelial cells (white bar); cells stimulated with thrombin in absence (black bar) or presence (grey bar) of TFP. (**B**) Activation of Ral in non-stimulated endothelial cells (left panel) and cells stimulated with thrombin in absence (second panel) or presence (right panel) of TFP. (**C**) Densitometric analysis of activation of Ral in the presence (gray bars) and absence (black bars) of TFP. On the *y*-axis fold stimulation of Ral activation compared to non-stimulated cells is given. On the *x*-axis time is depicted in minutes. Data from three independent experiments have been used to quantify activation of Ral in the presence and absence of TFP.

Overexpression of variant Ral and Rab3b in endothelial cells.

In the previous paragraphs we showed that Ral activation coincides with thrombin-induced release of VWF. To study the functional role of Ral in exocytosis of Weibel-Palade bodies, we expressed Ral wild type, constitutively active (GTP-bound) Ral G23V or dominant negative (GDP-bound) Ral S28N in primary human endothelial cells by electroporation. Expression of myc-tagged Ral G23V revealed that in the majority of transfected cells the number of Weibel-Palade bodies was greatly reduced (Fig. 3A-C).

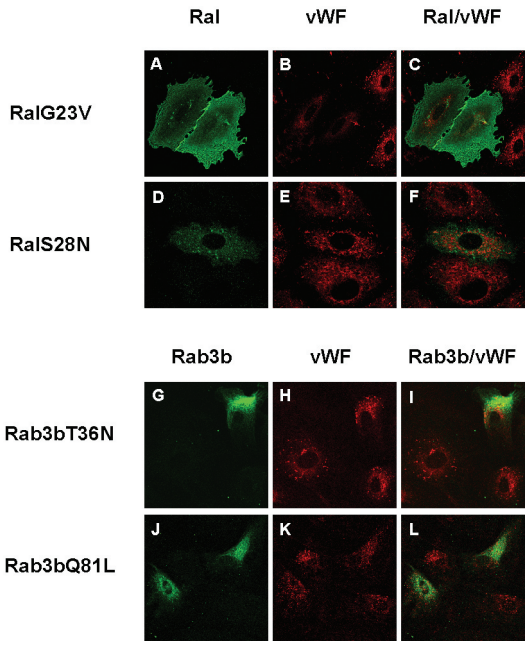

Figure 3. Wild type and constitutively active Ral induce exocytosis of Weibel-Palade bodies in endothelial cells. Primary endothelial cells were transfected with Ral and Rab3B variants and cultured on coverslips for 48 hours. Cells were fixed with 3.7% formaldehyde and immunofluorescence was performed as described in Materials and Methods. Exogenous Ral and Rab3B was visualized using monoclonal antibody 9E10 directed to Myc-tag and a FITC-labelled secondary goat anti-mouse antibody (**A, D, G, J**). VWF was visualized using polyclonal antibody directed to vWF and a Texas Red-labelled secondary horse anti-rabbit antibody (**B, E, H, K**). (**A, B, C**) Cells transfected with pCDNA3.1-Myc-Ral G23V; (**D,E,F**) Cells transfected with pCDNA3.1-Myc-Ral S28N; (**G,H,I**) Cells transfected with pCDNA3.1-Myc-Rab3B T36N; (**J,K,L**) Cells transfected with pCDNA3.1-Myc-Rab3B-Q81L.

In some of the transfected cells, a residual number of Weibel-Palade bodies could be detected (Table 1). Quantification of a large number of transfected cells revealed that 48% of cells expressing Ral G23V contained a limited number (<5) Weibel-Palade bodies (Table 1). A similar phenotype was observed in cells expressing wild type Ral (Table 1). In 48% of cells expressing wild type Ral the number of Weibel-Palade bodies is strongly decreased compared to non-transfected cells.

In cells transfected with dominant negative mutant Ral S28N normal amounts of Weibel-Palade bodies were observed. (Fig. 3D-F). Quantitative analysis revealed that in only 8% of cells transfected with Ral S28N reduced numbers of Weibel-Palade bodies were present. Similarly, 4% of non-transfected primary endothelial cells also contained reduced numbers of Weibel-Palade bodies. Overall, these results indicate that overexpression of Ral in the GTP-bound form reduces the number of Weibel-Palade bodies in endothelial cells. To provide evidence that cells express-

ing variant Ral are indeed endothelial cells, we performed co-localization studies with a monoclonal antibody directed against CD31, a transmembrane adhesion molecule which is abundantly expressed at intracellular junctions of endothelial cells [29]. CD31 was observed at the junctions of both non-transfected cells and cells expressing Ral G23V (data not shown). To further establish the specificity of the Ral G23V-mediated release of VWF from Weibel-Palade bodies, we expressed dominant negative (Rab3B T36N) and constitutively active Rab3B (Rab3B Q81L) in endothelial cells. Previously, Rab3B has been implicated in regulated exocytosis in neuroendocrine cells [30]. However, introduction of Rab3B T36N or Rab3B Q81L in human endothelial cells did not result in a decrease in the number of Weibel-Palade bodies (Fig. 3G-L). These findings indicate that expression of Rab3B does not induce exocytosis of Weibel-Palade bodies.

TABLE 1. Quantitative analysis of the number of Weibel-Palade bodies in transfected and non-transfected human endothelial cells.

	Number of Weibel-Palade bodies (n)		
Construct	n < 5	5 < n < 20	n > 20
non-transfected	4	48	48
Ral wild type	48	16	36
Ral G23V	48	33	18
Ral S28N	8	38	54

Primary endothelial cells were transfected with Ral wild type, Ral G23V, and Ral S28N as described in Materials and Methods. For each construct the number of Weibel-Palade bodies was determined in 20-30 transfected cells. Results are expressed in percentages and are compared to the number of Weibel-Palade bodies present in non-transfected cells.

DISCUSSION

Stimulation of endothelial cells with agents such as thrombin results in release of high molecular weight multimers of VWF and translocation of P-selectin to the plasma membrane. Both these events are an immediate consequence of the thrombin-induced exocytosis of endothelial cell-specific storage organelles, the Weibel-Palade bodies. In this report, we provide evidence that the small GTP-binding protein Ral is transiently activated following stimulation of endothelial cells by thrombin. A previous study has shown that activation of Ral occurs also upon stimulation of human platelets with thrombin [27]. In platelets, activation of Ral reached a maximum 1 minute following stimulation with thrombin whereas in endothelial cells maximal levels were reached after 2 min (Fig. 1) [27]. Furthermore, the amount of activated Ral decreased rapidly (between 5 and 10 min) in endothelial cells whereas in platelets significant levels of activated Ral were still present at 10

min following stimulation. Similar to what has been observed for platelets, elevation of intracellular calcium levels resulted in a rapid activation of Ral. In this study, we show that thrombin-induced activation of Ral is inhibited by the calmodulin antagonist TFP. This observation indicates that Ral functions downstream of calmodulin in endothelial cells. Also the release of VWF is decreased in the presence of TFP (Fig. 2). These findings lend additional support to a close correlation between activation of Ral and regulated secretion of VWF.

Functional involvement of Ral in regulated exocytosis of Weibel-Palade bodies by endothelial cells is suggested by the substantial decrease in number of Weibel-Palade bodies in endothelial cells overexpressing wild type and 'constitutively active' Ral. In some transfected cells, a residual number of Weibel-Palade bodies can still be detected (Table 1). This may relate to variability in expression levels of Ral variants among individual primary endothelial cells. A large variability in the number of Weibel-Palade bodies is also observed in non-transfected primary human endothelial cells (Table 1). A reduced number of Weibel-Palade bodies in a particular cell does not always result from exocytosis but may also be caused by cell-to-cell variability within primary cultures. Therefore, we determined the number of Weibel-Palade bodies in a large number of transfected primary endothelial cells. Our results suggest that both constitutively active Ral (G23V) and wild type Ral can induce exocytosis of Weibel-Palade bodies. The number of Weibel-Palade bodies in endothelial cells transfected with dominant negative Ral (S28N) is similar to control, non-transfected cells. We also studied whether dominant negative Ral interfered with stimulus induced exocytosis of Weibel-Palade bodies. Stimulation by thrombin, Ca^{2+}-ionophore A23187 and PMA did result in a decrease in the number of Weibel-Palade bodies in cells expressing Ral S28N similar to that observed in non-transfected cells (data not shown). These findings indicate that under our experimental conditions dominant negative Ral is unable to block stimulus-induced release of VWF from the Weibel-Palade bodies. Most likely the concentration of Ral S28N in transfected cells is too low to completely block cycling of endogenous Ral. Alternatively, Ral-independent signalling pathways may exist that are capable of inducing regulated exocytosis of Weibel-Palade bodies.

It should be noted that our analysis does not allow for direct monitoring of exocytosis of Weibel-Palade bodies. The amount of Weibel-Palade bodies in transfected cells is evaluated 48 hours following transfection. We cannot exclude that the observed effects of Ral on Weibel-Palade body content are not directly related to exocytosis. For instance, Ral may be involved in the formation of Weibel-Palade bodies from the trans Golgi network thereby reducing the number of these granules in cells transfected with active Ral-G23V. A possible role for Ral in granule biogenesis has recently been proposed. Several studies have suggested that a complex

of Ral with phospholipase D and Arf mediates vesicle budding from the Golgi apparatus [31,32].

In a previous study, we have reported that Ral associates with Weibel-Palade bodies in endothelial cells [18]. In the present study, expression of Ral variants did not reveal colocalization of Ral with VWF in Weibel-Palade bodies. Several reasons may be forwarded for this apparent discrepancy. First, Ral may only transiently associate with Weibel-Palade bodies. Finally, only a limited part of the intracellular amount of Ral may participate in the exocytosis of Weibel-Palade bodies. The intense membrane stain observed for Ral may interfere with the detection of small amounts of exogenous Ral that associate with Weibel-Palade bodies. Cell fractionation studies revealed that Ral is not exclusively present in subcellular fractions that contain Weibel-Palade bodies. A significant amount of Ral was detected in other subcellular fractions derived from endothelial cells (data not shown).

Several studies have suggested a role for Ral in cytoskeleton dynamics [19]. Ral interacts in a GTP-dependent manner with filamin inducing filopodia [33]. Furthermore, Ral interacts with RLIP76, a Ral effector protein with GTPase protein activity for cdc42 [20]. Inspection of Figure 5A-C reveals that cells expressing Ral G23V appear larger then surrounding endothelial cells. The observed morphological changes may result from an altered organization of the cytoskeleton in endothelial cells harbouring constitutively active Ral G23V. At present it is unclear whether the observed changes in organization of the cytoskeleton are related to the absence of Weibel-Palade bodies in cells expressing Ral G23V. Recently, it has been shown that thrombin induced release of VWF can be potentiated by specific inhibition of Rho, a small GTPase involved in cytoskeletal rearrangements like stress fiber formation [34]. This interesting observation suggests that the cytoskeleton may modulate agonist-induced release of Weibel-Palade bodies. It is possible that Ral-induced changes in the organization of the cytoskeleton may promote fusion of Weibel-Palade bodies with the plasma membrane.

Recently, Ral has also been implicated in endocytosis [35]. Interestingly, both constitutively active and dominant negative forms of Ral inhibited endocytosis. These observations suggest that GTP hydrolysis of active Ral is required for endocytosis. Potentially, Ral may be involved in the rapid endocytosis of integral membrane proteins like P-selectin and CD63, that are also contained within the Weibel-Palade bodies, following fusion of these organelles with the plasma membrane. Taken together, it appears that Ral may have multiple functional roles within endothelial cells. Future studies aim at defining individual steps in the biogenesis and release of Weibel-Palade bodies that are controlled by this small GTP binding protein.

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Chapter 5

VON WILLEBRAND FACTOR PROPEPTIDE IN VASCULAR DISORDERS

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ABSTRACT

Von Willebrand factor (VWF) is a multifunctional plasma protein that plays a prominent role in haemostasis. In endothelial cells processing of its precursor pro-VWF results in the formation of two large polypeptides, mature VWF and a propeptide. These proteins are co-secreted on an equimolar basis but are cleared from the circulation at different rates. VWF levels are frequently elevated in response to vascular disorders. Similarly, propeptide levels are increased under these conditions, although primarily in fulminant vascular disease, such as thrombotic thrombocytopenic purpura and septicemia. In chronic vascular disease, e.g. diabetes or peripheral vascular disease, propeptide levels are much less elevated. The differential response of VWF and propeptide levels to vascular disease could provide a means to assess the extent and time course of endothelial cell activation. After secretion, the propeptide may play a role in modulating cellular adhesion processes. Thus, enhanced propeptide secretion seems not of merely diagnostic significance.

INTRODUCTION

Von Willebrand factor (VWF) is a multifunctional, polyvalent plasma protein that plays a prominent role in events that lead to normal arrest of bleeding. VWF is synthesised by endothelial cells and megakaryocytes and originates from the VWF precursor pro-VWF, processing of which results in the formation of two large polypeptides, *i.e.* mature VWF (hereafter named VWF) and the VWF propeptide (also named von Willebrand antigen II, see below). Both polypeptides have distinct biological functions. VWF mediates the adhesion and aggregation of platelets at sites of vascular injury and also functions as a stabilising chaperone protein of factor VIII, an essential cofactor of the coagulation system. The VWF propeptide is required for proper intra-cellular, post-translational multimerisation and targeting of VWF to storage vesicles. Montgomery and Zimmerman were the first to identify VWF propeptide, called von Willebrand antigen II (VW AgII), a unique protein that, together with VWF, is deficient in plasma of patients with von Willebrand's disease [1,2]. After the full-length VWF cDNA was cloned, it became clear that VW AgII was identical to the propeptide moiety of the VWF precursor and, consequently, deficient in severe von Willebrand's disease [3-5].

To date most evidence supports that plasma VWF is predominantly derived from endothelial cells rather than from platelet storage pools [6]. Similarly, steady state plasma propeptide levels most likely stem from endothelial cells rather than from platelet stores [7]. As plasma VWF levels are increased in clinical conditions

associated with vascular abnormalities, such as coronary artery disease or peripheral vascular disease, it has been postulated that VWF could serve as a marker of endothelial cell perturbation or damage. Indeed, many studies have shown that plasma VWF levels are frequently elevated in disorders associated with endothelial cell dysfunction, including ischaemic heart disease, diabetes and inflammatory disorders. Raised plasma VWF concentrations may also have prognostic implications in vascular disease [8-13].

The significance of VWF as a specific marker of endothelial cell perturbation has also been challenged, however [6,13,14]. A major point of concern is the knowledge that the systemic VWF concentration does not necessarily reflect the VWF secretory capacity of endothelial cells due to extraction of VWF by subendothelial connective tissue. Secretion of VWF by endothelial cells occurs in a bi-directional fashion [15,16] and substantial amounts of released VWF may be trapped by the extra-cellular matrix [17,18]. In addition, secreted VWF may also bind to the endothelium itself [19,20], a process that may also mask VWF secretion. Furthermore, because of the relative long half-life of VWF in the circulation (about 12h), fluctuations in VWF levels will, in general, be less apparent. Also the latter might limit the use of VWF as an index of endothelial cell function.

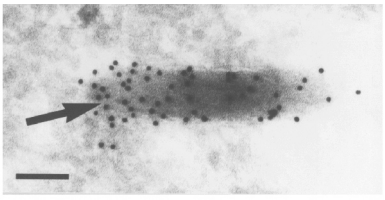
Recently, it was shown that measurement of VWF propeptide levels could provide a means to assess endothelial cell activation *in vivo* in a more specific and quantitative manner [5,14,21-23]. VWF propeptide is co- secreted from endothelial cells on an equimolar basis with VWF. However, unlike VWF, the propeptide is not trapped by subendothelial connective tissue after it is secreted [17]. Therefore, systemic propeptide levels probably more accurately reflect endothelial secretion processes than do VWF levels. Also from an analytical point of view measurement of propeptide levels for monitoring endothelial cell perturbation could be an attractive alternative. The three dimensional structure of the propeptide is by far less complex than the delicate quaternary structure of mature VWF. Consequently, in terms of accuracy and precision, propeptide analysis is probably less prone to analytical variations than VWF measurements.

Although the VWF propeptide could be an attractive candidate marker for endothelial cell perturbation *in vivo*, measurement of propeptide levels in combination with VWF levels could be of additional diagnostic value. This view stems from the observation that VWF and propeptide are cleared from the circulation at different rates [21]. It has been postulated that this feature could provide a means to assess the extent and time course of endothelial cell secretion rates and could allow for a more subtle and differentiated analysis of endothelial cell activation than reported before [14,21]. This review is intended to discuss the potential use of propeptide measurements as an index of endothelial cell function and to offer a perspective

on the significance of the VWF propeptide as a marker of endothelial cell perturbation.

CELLULAR BASIS OF VWF AND VWF-PROPEPTIDE RELEASE

Mature VWF and its propeptide are the end products of a complex biosynthetic pathway. (For comprehensive reviews on the cell biology and biochemical properties of VWF, see refs. [24,25]. Polymerisation of VWF is one of the most characteristic post-translational events that occurs during its travel to the outside of the cell (Fig. 1). This phenomenon is responsible for the typical polyvalent, multi-interac-



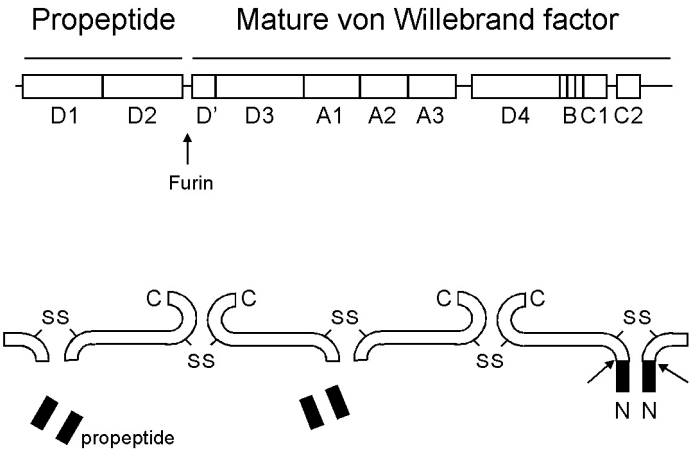


Figure 1. Assembly, processing and storage of VWF. The top panel shows an electron micrograph image of endothelial cells stained with a polyclonal antibody directed against VWF. Labeling of a rod-shaped vesicle (Weibel-Palade body) is observed (bar, 0.1 μm). The middle panel depicts a schematic representation of the primary structure of pro-VWF. The location of conserved structural domains and of the propetide and mature VWF moiety are indicated. The lower panel schematically depicts the multimeric structure of VWF. Pro-VWF monomers dimerise through cysteine residues located at the carboxy-terminus. Multimers are formed via cysteines in the D' and D3 domain. The propeptide plays an essential role in the assembly of multimers in that it promotes inter-chain disulphide formation and subsequent polymerisation [24,25]. The subtilisin-like endoprotease furin most likely catalyses the cleavage (arrows) of the propeptide portion of the molecule [73-75]. The propeptide is probably secreted as a dimer [19]. (Courtesy of dr. Hubert de Leeuw, ref.76).

tive nature of VWF. The propeptide moiety of pro-VWF serves a major role in this process. It both controls inter-subunit interaction through homo-dimerisation of the amino-terminal regions of two pro-VWF molecules and catalyses the formation of disulphide bonds between them [26,27]. After endoproteolytic cleavage of pro-VWF in the *trans*-Golgi network [28], mature VWF and its propeptide partition between two different secretion pathways. The majority of VWF and propeptide molecules that are generated upon proteolytic maturation is directly secreted by endothelial cells, together with some unprocessed pro-VWF (Fig. 2, [29]). A relatively small portion is co-stored in unique cytoplasmic vesicles, the Weibel-Palade bodies. The latter pool, representing mature and functionally the most potent VWF multimers, is only secreted upon proper stimulation of the cell. For instance, when cultured endothelial cells are treated with phorbol myristate acetate (PMA), a phorbol ester known for its ability to stimulate the protein kinase C-dependent signal transduction pathways, VWF and propeptide are released rapidly and in essentially equimolar amounts (Refs.14,21; Fig. 3). Similarly, more physiological secretagogues that give rise to an increase of cytosolic free Ca^{2+}, such as thrombin or histamine, or exposure of endothelial cells to cAMP-raising agents, such as epinephrine, adenosine or prostacyclin, rapidly induce exocytosis of VWF from Weibel-Palade bodies [30-35]. Although this has not been studied in detail, it is to be expected that also the VWF propeptide is released under these conditions through the regulated pathway.

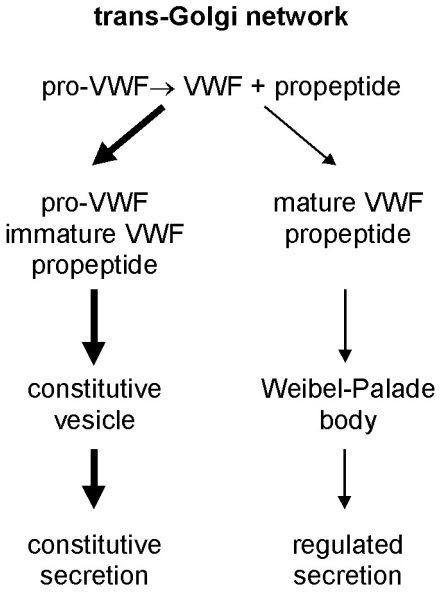


Figure 2. Partitioning of VWF, propeptide and pro-VWF between the constitutive and regulated secretory pathway by vascular endothelial cells. Pro-VWF is processed in the trans-Golgi network and results in heterogeneous forms of VWF, ranging from dimers to large polymers (see also Fig. 1). The majority of the VWF species, consisting of partially processed, immature VWF is secreted through the constitutive pathway. The remainder, the largest, fully processed and functionally mature VWF, is sorted into Weibel-Palade bodies and secreted upon demand. This model proposes that the majority of VWF and its propeptide are secreted through the constitutive pathway [29].

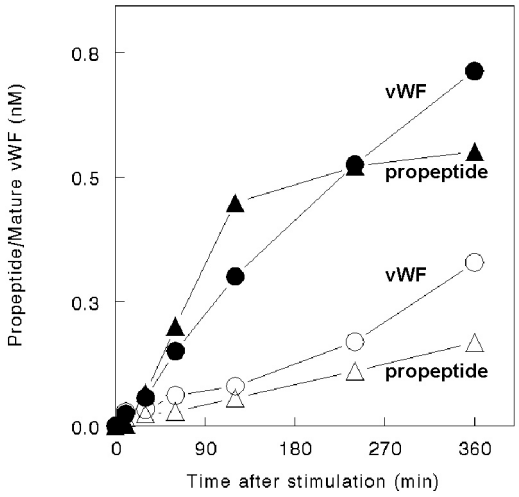


Figure 3. Regulated secretion of VWF and its propeptide by endothelial cells. Human umbilical vein endothelial cells were stimulated with the phorbol ester PMA (50 ng/ml) and at indicated time points VWF and propeptide levels were measured. Open symbols, release of VWF and propeptide by quiescent endothelial cells; closed symbols, secretion of VWF and propeptide induced by PMA. Note that VWF and propeptide are secreted in essentially equimolar amounts.

Besides VWF and its propeptide, Weibel-Palade bodies have been shown to contain a subset of other proteins, including P-selectin, interleukin-8 (IL-8) and endothelin (Table 1). Similar to VWF and the propeptide, these proteins are transported to the outside of the cell upon stimulation and may control local or systemic biological effects, including inflammatory and vaso-active responses. If one considers endothelial cell perturbation and subsequent exocytosis of Weibel-Palade bodies as an etiological component of vascular disease, this feature should be taken into account as well.

Table 1. Residents of Weibel-Palade bodies

	References
Mature vWF	[47,61,62]
VWF propeptide	[17,62]
P-selectin	[63-65]
CD63	[66]
Endothelin	[67]
Interleukin-8	[68,69]
α 1,3-fucosyltransferase VI	[70]
Tissue-type plasminogen activator	[71,72]

VWF PROPEPTIDE SECRETION IN VIVO

As a plasma constituent, the VWF propeptide is a rather abundant protein [7,14,21,22,36-38]. In healthy individuals the propeptide concentration ranges from about 2 to 10 nM (Fig. 4). Assuming a molecular mass of 100 kDa, this corresponds to an average concentration of about 0.6 µg per ml. As expected, in subtypes of von Willebrand's disease characterised by a major gene defect (*e.g.* von Willebrand's disease type 3) no propeptide is detectable whereas in milder cases of this disorder, such as von Willebrand's disease type 1, propeptide levels are reduced, though to an unpredictable extent [2,37]. Of interest is the observation that, unlike VWF, propeptide concentrations are not, or to a much lesser extent, dependent on ABO blood type [21,37,39]. Most likely, the propeptide does not carry the ABO blood group antigens that have been described for VWF [40-42]. Consequently, differences in blood group specificity will not complicate the evaluation of propeptide levels and may also explain why the correlation between peripheral VWF and propeptide levels is rather poor, at least in healthy individuals (Fig. 4, [2,14,37]).

Another point of interest is that the molar ratio of the propeptide and mature VWF is about 0.1 under resting conditions. This value is clearly lower than the equimolar ratio observed in cultured endothelial cells ([22], Fig. 3). Because the

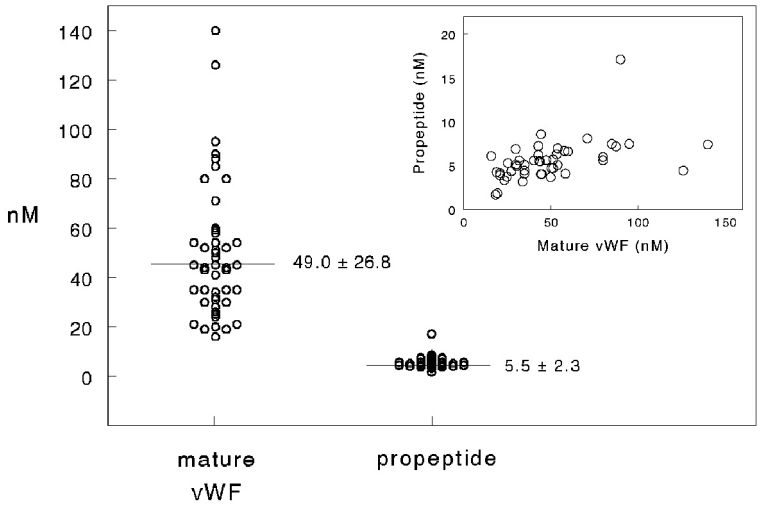


Figure 4. Concentration of propeptide and mature VWF in normal plasma. The concentration of VWF (mean concentration ± 2SD of 47 healthy individuals) is expressed as the half-homodimer concentration, assuming that normal pooled plasma contains 10 µg VWF/ml (50 nM). The insert shows the concentration of VWF and propeptide in the individual plasma samples. (From Borchiellini *et al.* [21], with permission).

propeptide disappears four to five times faster from the circulation than mature VWF, it seems reasonable to assume that the observed differences in steady state concentration are due to differences in half-life. Pertinent to this point is the observation that upon perturbation of the endothelium, for instance elicited by experimental disseminated intravascular coagulation (DIC), or administration of 1-deamino-8-D-arginine (desmopressin, DDAVP), propeptide levels return much faster to their baseline values after termination of the vascular challenge than VWF levels ([7,14]; Fig. 5).

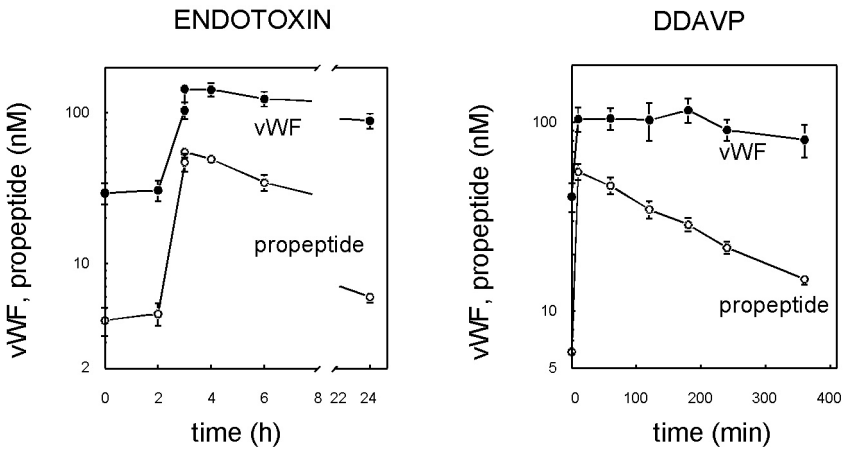


Figure 5. Effect of experimental endotoxemia and administration of DDAVP on VWF and propeptide levels in healthy individuals. Endotoxin or DDAVP were administered at time 0 and at the time points indicated propeptide and VWF levels were measured. [From van Mourik *et al*. [7], with permission].

VWF PROPEPTIDE SECRETION IN VASCULAR DISEASE

A number of studies have shown that propeptide levels are not, or only slightly elevated in patients with a long history of vascular disease, such as peripheral occlusive vascular disease or chronic diabetes mellitus (Table 2, Fig. 6). Propeptide levels do, however, correlate with those of VWF. These studies show that in *chronic* vascular disease the propeptide is not a more sensitive marker of endothelial cell dysfunction than VWF levels. On the other hand, in cases of frank, acute vascular disease as may occur in thrombotic thrombocytopenic purpura (TTP) or septicemia, both propeptide and VWF levels are clearly elevated. This pattern can readily be explained in terms of acute perturbation of the endothelium. These data suggests that propeptide analysis adds an extra dimension to the diagnostic evaluation of vascular diseases. Measurement of both propeptide and VWF levels allows to discriminate between chronic and acute phases of endothelial cell activation and

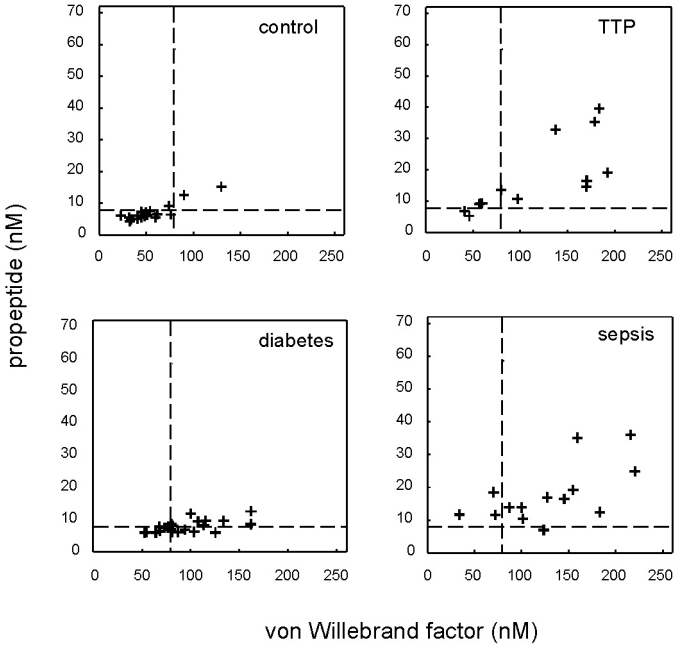


Figure 6. Relation between plasma pro-peptide and VWF levels in patients with acute (TTP, septicemia) and documented chronic vascular disease (diabetes). Dotted lines represent the upper limit of the 95% confidence interval of respectively VWF and propeptide levels of the control group. The latter group consisted of patients without vascular disease admitted to the hospital. (From van Mourik *et al.* [7], with permission).

provides a basis for a more differentiated analysis of endothelial cell activation that may occur in response to vascular pathology.

The interpretation of propeptide data might be complicated by the contribution of platelet propeptide to elevated propeptide levels, particularly in clinical situations associated with massive platelet activation such as TTP and overt septicemia. Probably this is not, or only to a limited extent, the case. First, there is no clear relationship between platelet number and propeptide level, neither in healthy individuals, nor in patients with thrombocytemia or thrombocytopenia [7]. Finally, there is no clear correlation (J.A. van Mourik, R. Fijnheer, unpublished observations) between propeptide concentrations and plasma levels of soluble P-selectin, a specific marker of platelet activation [44,45]. Second, the amount of propeptide stored in the α-granules of platelets is probably not sufficient to account for the increased propeptide concentrations [14,21,37].

ORIGIN OF VWF PROPEPTIDE AND VWF IN VASCULAR DISEASE

As noted above, cultured endothelial cells release the majority of *de novo* synthesised VWF, either partly or completely processed, through the constitutive pathway

Table 2. Propeptide and VWF levels in vascular disease[ϕ)]

Disease	Propeptide (%)	VWF (%)	n	Reference
Acute				
Thrombotic	440[#)]	ND	9	43
thombocytopenic purpura				
	243.7**[)]	207.9**[)]	13	7
Haemolytic uremic	427[#)]	ND	5	43
syndrome				
Sepsis	250.7**[)]	235.8**[)]	14	7
Chronic				
Peripheral vascular	160.5*[)]	141.8*[)]	24	22
disease				
	104.8[§)]	110.6[§)]	20	38
Ischaemic heart disease	149.6*[)]	145.1*[)]	25	22
Diabetes	112.7[(NS)]	174.4**[)]	22	7
	147.8**[)]	200.9**[)]	19	23

[ϕ)] Data are collected from 5 different studies. Mean values are listed as % of normalised control values (100%), unless otherwise indicated.
*) $p < 0.05$, **) $p < 0.001$; n, number of patients studied.
ND, not reported; NS, not significant; [§)] median value; [#)] significance not reported.

[29]. Under these conditions, only part of the stored propeptide and functionally mature VWF is secreted through the regulated pathway. Whether secretion of VWF and propeptide occurs in a similar way *in vivo*, remains to be established. To what extent the plasma VWF and propeptide pool stems from either the constitutive or the regulated pathway is unknown. Only trace amounts of pro-VWF, a candidate marker of the constitutive secretion pathway, are detectable in plasma [21]. This suggests that under normal physiological conditions the constitutive pathway does not substantially contribute to the release of VWF. On the other hand, it is possible that upon release, pro-VWF is rapidly cleared from the circulation, or, alternatively converted into mature VWF and propeptide outside the cell. Indeed, experimental studies in animals have shown that upon infusion of (unprocessed) pro-VWF, its propeptide is rapidly cleaved from the precursor protein [46].

It should be noted that the storage capacity of endothelial cells is limited. Particularly in cases where the endothelium is exposed to repeated challenges, such as in chronic vascular disease, the propeptide and VWF pool might be exhausted. In cultured endothelial cells it takes about one to two days after a single exposure to endothelial cell agonists to restore this pool [47]. Taking into account that only a fraction of newly synthesised pro-VWF is directed to Weibel-Palade bodies [29], it is to be expected that in situations of severe and widespread endothelial cell per-

turbation, such as may occur in TTP or septicemia, released propeptide and VWF primarily originate from the Weibel-Palade bodies. Pertinent to this point is also the observation that in the acute phase of myocardial infarction the plasma concentration of high molecular weight VWF multimers increased [48]. As these large VWF polymers primarily stem from endothelial cell Weibel-Palade bodies [29], the raised VWF levels observed in these clinical situations most likely originate from the regulated pathway, at least in part.

Persistent rises of plasma VWF and propeptide most likely reflect enhanced constitutive release. The latter condition would require upregulation of *de novo* pro-VWF synthesis. Indeed, under various conditions endothelial cell-specific VWF synthesis can be subject to enhanced transcriptional activity [49,50]. It seems reasonable to assume, therefore, that elevated propeptide and VWF levels in patients with sustained endothelial cell injury not only reflect regulated secretion but also increased transcriptional activity and subsequent enhanced constitutive release. In any case, only mature VWF and its propeptide are detectable in plasma in significant amounts but not their precursor pro-VWF. Knowledge of the contribution of either pathway in supplying functionally mature VWF multimers, its precursor pro-VWF and its propeptide to the blood stream, could be of help to get insight into the complex functional alterations of the endothelium either as a cause or a result of vascular disease.

BIOLOGICAL SIGNIFICANCE OF PROPEPTIDE RELEASE

Evidence is accumulating that the VWF propeptide not only plays an important role in controlling intra-cellular targeting and polymerisation of VWF, but may also serve a variety of biological functions after its release from the cell, notably in inflammatory and cell adhesion processes. In this respect, enhanced secretion of the propeptide in various clinical situations is not merely of diagnostic significance. Recent studies indicate that the propeptide, apart from its putative inherent protein disulphide isomerase (PDI) activity [51], could serve a role in controlling cell adhesion processes by various mechanisms. For instance, it has been shown that the VWF propeptide is a substrate for coagulation factor XIIIa. This transglutaminase is able to catalyse the cross-linking of the propeptide to laminin, a constituent of the extra-cellular matrix [52,53]. As laminin has a variety of cellular functions, including its role as cell attachment or cell migration mediator, it is tempting to speculate that the propeptide could play a role in modulating these processes.

Of particular interest is also the observation that the propeptide harbours a recognition site for $\alpha 4\beta 1$ (VLA4) and $\alpha 9\beta 1$ (VLA9) and is able to promote specific

adhesion and spreading of certain leukemia and melanoma cell lines mediated by these cell surface receptors [54-56]. Interestingly, these integrins also bind factor XIIIa. Both integrins are highly expressed on leukocytes; VLA-4 is primarily expressed by lymphocytes, monocytes and eosinophils, whereas VLA-9 is mostly found on neutrophils [57]. As these integrins play a prominent role in transendothelial migration of these cells, the propeptide could modulate these processes. It has also been shown that the propeptide may bind to collagen (type I) and inhibits collagen-induced platelet aggregation [58,59].

Taken together, it seems clear that the propeptide may serve a number of extra-cellular functions and is not just a waste product after it is secreted. This is reminiscent of proinsulin C-peptide, the propeptide of insulin which, like the VWF propeptide, participates in inter-chain disulphide bond formation necessary for generating mature insulin. After its secretion, along with insulin, C-peptide appears to produce significant biological effects, including restoration of diabetes- and hyperglycemia-induced vascular and neural dysfunction [60].

CONCLUSION AND FUTURE PERSPECTIVES

This review has served to illustrate the potential role and significance of the VWF propeptide as a novel marker of endothelial cell perturbation. This is a rather young field of investigation and clearly more studies are required to reveal the significance of the propeptide in vascular biology. From a clinical perspective, prospective, serial studies should reveal whether indeed measurements of both propeptide and VWF levels have a predictive value in terms of disease activity and are useful in monitoring the degree of vascular involvement as well as the response to therapy. It would also be of great interest to get insight into the biological and pathophysiological significance of the regulated and constitutive VWF secretion pathway. Through each pathway different VWF species are secreted. These VWF molecules differ in degree of polymerisation and adhesive properties and knowledge of the contribution of each pathway in providing VWF (and its propeptide) to the extra-cellular milieu, could lead to a better understanding of the role of the endothelium in controlling VWF-mediated processes in vascular pathology. This is still a rather poorly understood phenomenon. Finally, it also seems clear that the biological function of the propeptide is more diverse than previously anticipated. It will be a challenge to determine the physiological significance of this aspect of VWF biology in more detail.

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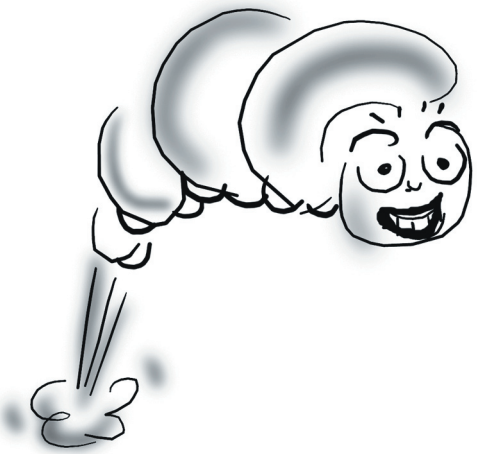
Chapter 6

ENDOTHELIAL CELL DAMAGE IS NOT THE PRIMARY EVENT IN TTP



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ABSTRACT

It is generally assumed that endothelial cell injury is the primary event in the pathogenesis of TTP. In this study we have determined the extent of vascular perturbation during acute episodes of the disease. We performed a prospective, serial study of 9 patients with relapsing TTP during hospitalization and treatment and assessed the degree of endothelial cell involvement at admission, exacerbation and remission by measurement of VWF and VWF-propeptide levels. Measurement of both VWF and its propeptide allows discrimination between acute and chronic perturbation of the endothelium. Elevated levels of both VWF and propeptide were found at admission. These levels decreased immediately upon plasma exchange therapy. However, plasma VWF and propeptide concentrations did not change, even at exacerbations. These observations indicate that endothelial cell activation is not the primary event leading to TTP. Vascular perturbation seems rather a consequence than a cause of the disease.

INTRODUCTION

Thrombotic thrombocytopenic purpura (TTP) is a rare hematological disorder that appears mostly at young age, of acute presentation and difficult diagnosis. The clinical features are thrombocytopenia, microangiopathic hemolytic anemia, renal failure, and neurological abnormalities resulting from formation of platelet thrombi within the microvasculature [1-4]. However, the etiology of the formation of platelet aggregates remains elusive. So far, accumulated data suggest that two important risk factors are involved in the events leading to the manifestation of TTP [5]. It is generally assumed that endothelial cell injury is one of the initial events in the pathogenesis of TTP. This view is primarily based on the observations that, in TTP, endothelial cells of the microvasculature are prone to apoptosis [6]. In addition, VWF multimers present in plasma of patients with TTP are sometimes larger than VWF multimers found in normal plasma. These multimers most likely stem from Weibel-Palade bodies which release their contents upon endothelial cell perturbation [7-9]. These VWF species are more potent in controlling platelet adhesion and aggregation than normal VWF multimers [10-12] and, as such, may contribute to pathologic platelet aggregation and arteriolar thrombi formation. A second risk factor thought to be involved in TTP is deficiency of the VWF-cleaving protease ADAMTS13 [13,14], a proteinase that has recently been identified as a member of the ADAMTS family of metalloproteinases [15-17]. Deficiency of this protease is thought to cause accumulation of the unusually large and highly reactive multimeric forms of VWF

that can adhere disproportionally to platelets and increase their adhesive potential. Evidence for constitutive protease deficiency as well as for deficiency associated with autoimmune inhibitors of this protease has been provided [14,18].

To document in more detail whether endothelial cell perturbation is the primary event in TTP, we performed a prospective, serial study of patients with relapsing TTP during hospitalization and treatment. To our knowledge, prospective studies on the role of endothelial cells in TTP have not been reported. To measure the degree of vascular involvement in TTP we examined levels of both plasma VWF and VWF-propeptide at various stages of the disease. The rationale of this approach is based on the observation that measurement of both VWF and propeptide plasma levels allows discriminating between chronic and acute phases of endothelial cell activation [19-21]. In patients with a long history of vascular disease, such as peripheral occlusive vascular disease or diabetes mellitus, propeptide levels are not, or only slightly, elevated, whereas VWF levels are increased two- to three-fold. On the other hand, in cases of acute vascular disease, as may occur in TTP, both propeptide and VWF levels are clearly elevated. On the basis of these observations, it was postulated that measurement of both propeptide and VWF levels allows discrimination between acute and chronic perturbation of the endothelium in TTP. So far, these data concerning the involvement of the endothelium in TTP have been obtained in a cross-sectional study [20]. We reasoned that only a prospective study could reveal whether indeed measurements of both propeptide and VWF levels have a predictive value in terms of disease activity and are useful in monitoring the degree of vascular involvement as well as the response to therapy in TTP. Therefore, we studied TTP patients during hospitalization and treatment and focussed on patients suffering from one or more exacerbations. This allowed assessing the degree of endothelial cell perturbation also at this critical phase of the disease.

MATERIALS AND METHODS

Patients

From 21 patients that were admitted to the hospital with acute symptoms of TTP, 9 having one or more exacerbations (relapsing TTP following initial complete response) were included in this study. Upon admission to the hospital, all patients had thrombocytopenia (platelet count <100 x 10^9/L; normal platelet count: 150-450 x 10^9/L) and microangiopathic hemolytic anemia (lactate dehydrogenase (LDH) levels > 800 U/L; normal LDH levels: 300-620 U/L). Most of the patients had a decreased hemoglobin level and 3 of them had impaired renal function (creatinine > 100 µmol/L). No VWF-protease activity was found in any of the patients upon admission. The clinical data at admission to the hospital are listed in Table 1.

Treatment

All patients were daily treated with plasma exchange for at least eight days or until a complete response (platelet count > 100 x 10^9/L and serum LDH < 800 U/L, for at least two consecutive days) was established. The frequency of plasma exchange was then decreased to 3 times a week and finally 2 times a week. Plasma exchange was immediately restarted upon exacerbation (thrombocytopenia and hemolytic anemia following initial complete response). During plasma exchange, one and a half plasma volume of the patient was removed daily and replaced by the same volume of fresh frozen plasma. Remission is defined as 30 days or more of complete response [4].

Blood collection and assays

Blood was sampled daily during plasmapheresis and at exacerbation. The frequency of blood sampling was decreased at complete response and remission. Blood samples were taken both before and after plasma exchange. All samples used for this study were obtained after informed consent. Blood was collected by vacutainer system in 3.1% citrate (1:10). To obtain platelet-free plasma, the blood was centrifuged for 15 minutes at 4°C at 2000 g, the supernatant was removed and centrifuged a second time. Samples were stored at -80°C. VWF and VWF-propeptide concentrations were measured by enzyme-linked immunosorbent assay (ELISA) as described previously [22]. Normal plasma from a pool of 30 donors served as standard. This plasma pool contains 6.3 nmol/L VWF-propeptide as assessed by calibration against purified recombinant propeptide [22] and 50 nmol/L of VWF.

VWF cleaving protease

VWF-cleaving protease (CP) activity was quantified by using an assay based on the preferential binding of high-molecular-weight forms of VWF to collagen [23]. Recombinant VWF with a substitution of glutamine for arginine at amino acid position 834 (VWF R834Q), which has an enhanced sensitivity to proteolysis by the protease, was used as substrate. The construction, expression, and purification of recombinant human VWF R834Q was described previously [24,25].

Statistical analysis

Statistics were performed using SPSS software version 10.0 (SPSS, Chicago, IL, U.S.A.). Data are presented as mean ± SD. Differences between groups were analyzed by ANOVA, followed by Tukey post hoc tests. Correlations between VWF and propeptide levels were determined by bivariate correlation analysis (Pearson). P values < 0.05 were considered to represent statistically significant differences.

RESULTS

Patient characteristics

Nine patients with acute symptoms of TTP and one or more exacerbations were followed in time during hospitalization. All patients suffered from thrombocytopenia (mean platelet count, 10 x 10^9 platelets/L; range, 2 – 21 x 10^9 platelets/L, Table 1) and severe hemolysis (mean LDH level, 5030 U/L; range, 2275 - 10780 U/L). Three of the nine patients had impaired renal function (creatinine > 100 μmol/L). At admission, mean levels of VWF (100.9 ± 26.2 nM) were twice as high as the VWF values of healthy individuals (50 ± 25 nM). VWF-propeptide concentrations were also significantly increased (21.0 ± 11.6 nM). All patients at hospitalization had no detectable VWF cleaving protease activity in their plasma. In four of them an inhibitor of the protease was detected. This indicates that some patients may suffer from congenital TTP, whereas others had developed inhibitory antibodies against the VWF-CP.

Table 1. Presenting features of patients with TTP

Patient number	Age (years)	number of exacerbations during study	LDH (U/L)	creatinine (μmol/L)	Hb (mmol/L)	platelets (x10^9/L)	VWF (nM)	propeptide (nM)	CP activity admission (%)	CP inhibitor admission	CP activity remission (%)
Normal (range)			330-620	58-103	7.4-9.6	150-450	25-75	4-10	>50		
1	30	1	6409	123	3.8	21	85.1	34.8	<5	-	80
2	54	2	2608	87	5.4	12	98.1	15.2	<5	-	0
3	29	3	6750	96	6.2	5	107.1	26.6	<5	-	9
4	22	1	3022	378	6.2	4	108.1	15.3	<5	+	100
5	24	2	3133	91	5.3	11	84.1	9.4	<5	+	80
6	30	1	10780	109	6.2	2	160.3	36.8	<5	+	70
7	26	2	4735	67	2.9	6	95.9	13.1	<5	-	5
8	42	1	2275	75	7.5	14	64.9	5.9	<5	-	5
9	31	1	5556	92	6.2	12	104.2	31.8	<5	+	100
average	32		5030	124	5.5	10	100.9	21.0			

Abbreviations: CP, VWF-cleaving protease
CP inhibitor: absent (-), present (+)

LDH and platelets levels during exacerbation and remission

All patients were treated with plasma exchange. The initiation of plasma exchange was associated with an immediate drop of LDH levels in all patients. LDH levels reached normal values within 8 days, provided that no exacerbation occurred. Upon treatment, platelets levels increased gradually, reaching values greater than 100 x 10^9 /L within one week after treatment. Despite first recovery, all patients included in this study suffered from one or more exacerbations during treatment (Table1), defined by recurrent hemolysis and thrombocytopenia. In most of the patients,

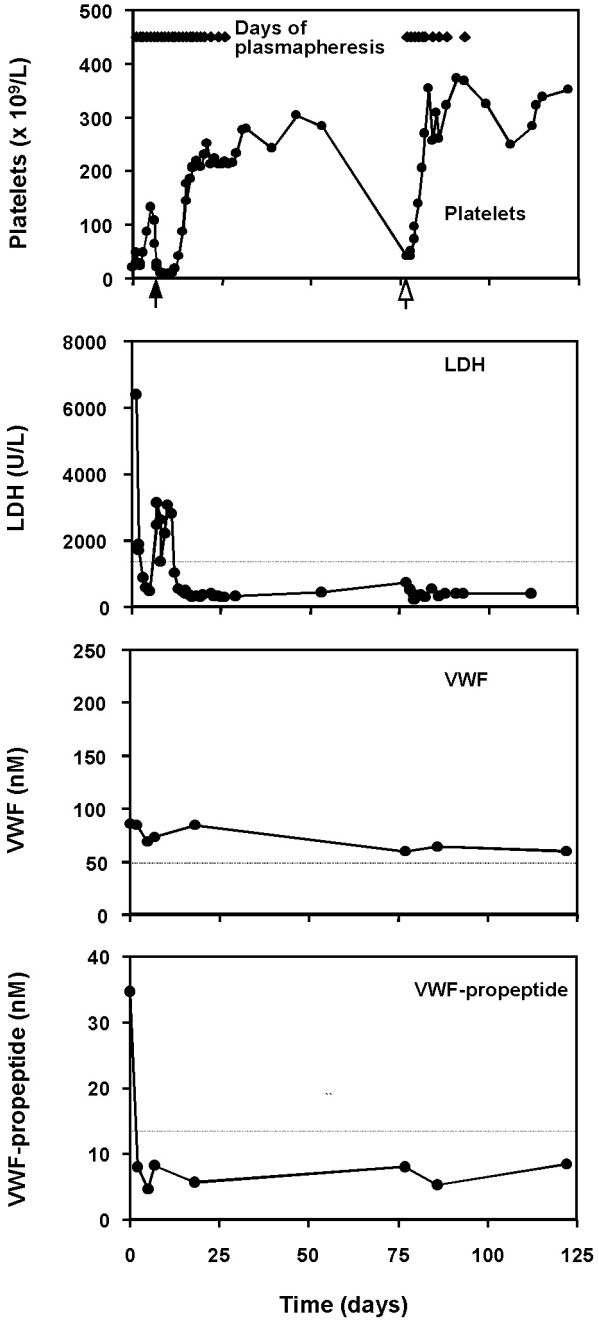

Figure 1. Time course of platelet count, LDH, VWF and VWF-propeptide levels in a patient with relapsing TTP during hospitalization.
On admission, the patient suffered from thrombocytopenia (21 x 10^9 platelets/L) and severe hemolysis (LDH > 6000 U/L). Upon plasma exchange therapy (indicated by filled circles) platelets and LDH levels began to recover slowly (days 4-6). Thrombocytopenia and hemolysis recurred at day 7 (exacerbation, filled arrow). After extensive therapy, platelets and LDH recovered and remained normal until remission was established (day 45). At day 77, the patient relapsed a second time (open arrow) and recovered after treatment. Also VWF and propeptide levels were elevated at admission (85.1 and 34.8 nM respectively). Upon plasmapheresis, propeptide levels decreased immediately reaching normal values (day 2). VWF levels decreased as well, though slower. Propeptide and VWF levels remained stable during further hospitalization, even at exacerbation (day 7 and 77). Dotted lines represent the normal levels.

exacerbation occurred when the intensity of plasma exchange was diminished. Because of exacerbation, daily plasma exchange was reinitiated until LDH and platelets returned to normal levels. Remission was established when these two parameters stabilized at normal values for 2 or more days. Figure 1 depicts the follow up of one patient during treatment.

Figure 2 shows the mean levels of LDH and platelets at hospitalization, at exacerbation and during remission. During remission, patients had normal concentrations of LDH (418 ± 87 U/L) and platelets (278 ± 73 x10^9/L). These data were significantly different from values measured at admission ($p<0.01$ and $p<0.001$ respectively). At exacerbation, the levels of LDH were elevated (1298 ± 822 U/L) but not significantly different from remission values. LDH levels at exacerbation were, however, significantly lower than at hospitalization ($p<0.01$). Similarly, the number

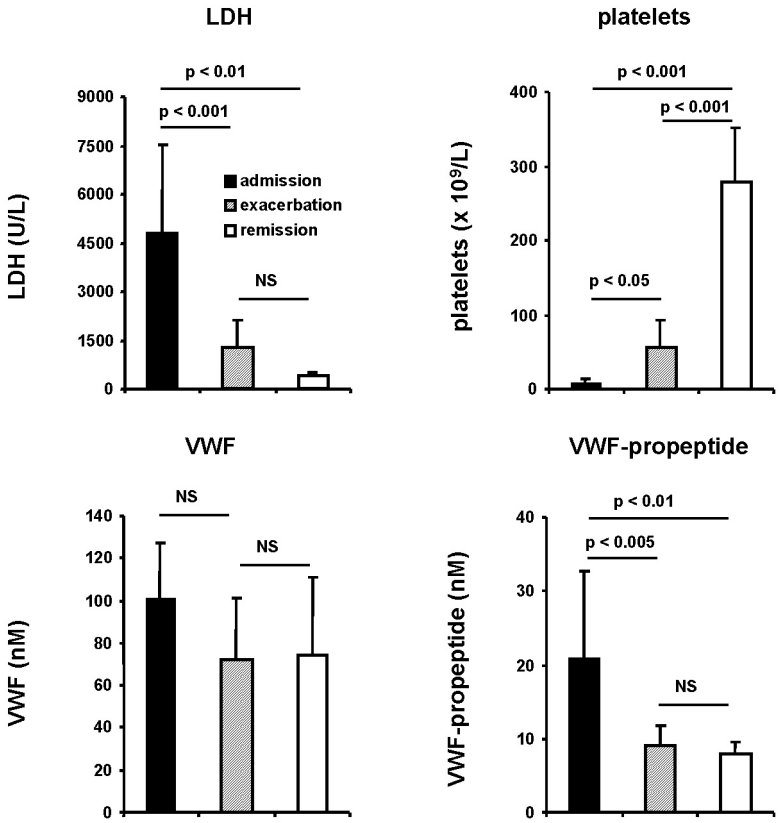

Figure 2. Mean levels of LDH, platelets, VWF and propeptide at admission, exacerbation and remission.
Admission (all parameters, n=9), exacerbation (LDH, n=13; platelets, n=14; VWF and propeptide, n=12), remission (LDH, n=3; platelets, VWF and propeptide, n=5). NS, Not Significant.

of platelets at exacerbation was low (56 ± 37 x10^9/L) compared to remission levels ($p<0.001$). However platelet number was significantly higher than at admission ($p<0.05$). The differences in LDH and platelets levels at exacerbation compared to those at hospitalization were probably due differences in clinical management. As soon as platelets dropped and LDH increased, patients were submitted to daily plasmapheresis, resulting in less extreme values of these two parameters. These observations confirm that upon treatment, LDH and platelets levels recover.

VWF and propeptide levels during exacerbation and remission

Upon plasma exchange VWF levels either decreased slightly (7 patients) or remained elevated (2 patients) (cf. Fig.1). On the other hand, propeptide levels dropped immediately in all patients upon plasmapheresis, reaching normal values in 8 of the 9 cases. During exacerbation, which was associated with an increase of LDH and a decrease of platelet number, both VWF and propeptide levels remained stable (cf. Fig. 1). Also at remission VWF and propeptide concentrations did not change.

Figure 2 shows that VWF levels at exacerbation (71.8 ± 29.2 nM) were lower than VWF levels at admission (100.9 ± 26.2 nM). However, the difference was not significant. During remission, VWF levels remained elevated (74.1 ± 36.7 nM) and did not significantly differ from admission and exacerbation values. Thus, TTP patients have, also outside the acute phase of the disease, significantly elevated VWF levels. The propeptide pattern during various phases of the disease was distinct from the VWF pattern (Fig. 2). Unlike at admission, propeptide concentrations were not elevated at exacerbation (9.1 ± 2.8 nM, $p<0.01$). Propeptide levels during remission remained normal (8.0 ± 1.6 nM).

In Figure 3, propeptide and VWF levels during various stages of the disease are depicted on an individual basis. This figure shows that at admission both VWF

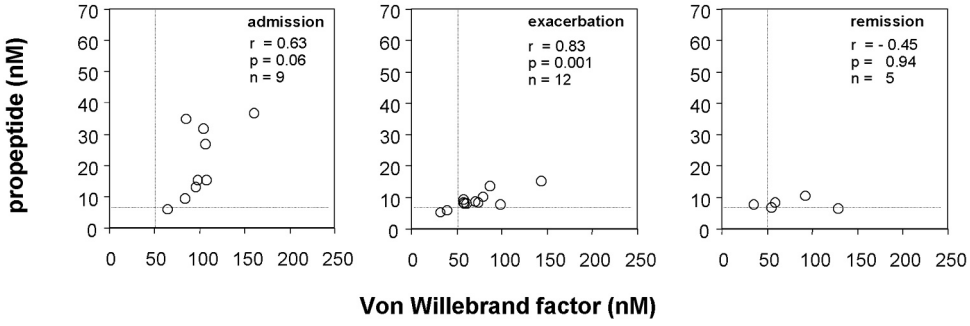

Figure 3. Relation between plasma VWF and propeptide levels in individual patients with TTP at admission, exacerbation and remission. Dotted lines represent the normal levels of VWF and propeptide.

and propeptide levels are elevated. In contrast, at exacerbation and remission most of the patients had normal propeptide concentrations but elevated VWF levels.

DISCUSSION

It is generally believed that damage or activation of endothelial cells is a prominent event in the pathogenesis of TTP. The present study provides evidence that acute perturbation of the endothelium is less evident in overt, relapsing TTP than previously anticipated. Endothelial activation seems rather a secondary event. This conclusion is primarily based on the observation that during hospitalization and excessive treatment of patients with chronic relapsing TTP, exacerbations that occurred during the follow up period did not result in elevation of plasma levels of the propeptide of VWF. Similarly, VWF levels did not significantly change during this acute phase of the disease. As elevated VWF-polypeptide levels reflect fulminant, acute vascular disease [19,20,22], one would expect that exacerbation of the disease activity would be associated with increased propeptide levels (and concurrent increase of plasma VWF). This appeared not to be the case. In only 2 of the patients that experienced an exacerbation, propeptide levels increased (Fig. 3). On the other hand, at hospitalization, both propeptide and VWF levels were significantly elevated, a finding which, as also shown previously [20,21], is indicative of vascular involvement in the pathogenesis of the disease. This is further corroborated by the observation that successful response to plasma exchange treatment, as demonstrated by normalization of platelet count, LDH level and clinical signs of the disease, was also associated with normalization of propeptide levels and reduction of VWF levels (though not significantly) in 8 out of the 9 patients studied. We can only speculate about the discrepancy observed between VWF and VWF-propeptide levels at admission and further hospitalization. It seems reasonable to assume that propeptide and VWF levels of plasma collected immediately upon admission to the hospital reflect vascular stimulation for a prolonged, though unknown period of time. In contrast, exacerbations were treated immediately by plasma exchange. We suppose that rapid and adequate therapeutical intervention has prevented the occurrence of new vascular events, such as secretion of propeptide and VWF. Vascular perturbation observed at hospitalization seems rather a consequence than a cause of TTP.

The immediate drop of plasma levels of propeptide upon plasmapheresis at admission and the long-term normalization of propeptide levels suggest that plasma exchange therapy suppresses regulated release of the propeptide, and as VWF and propeptide are stored together in Weibel-Palade bodies, also of VWF.

Apparently, circulating agonists that trigger release of VWF and its propeptide from the endothelium are neutralized during plasma exchange therapy. However, the effect of plasma exchange on VWF levels is less prominent than on the propeptide levels. It seems likely that this is due to the differences in half-life between VWF and propeptide (respectively 12 and 3 hours) [20]. These observations further support the view that the VWF-propeptide is a better marker for endothelial cell perturbation than VWF [19,20]. This study also indicates that measurement of the propeptide may provide an adequate index for the effect of therapy on the excitation state of the endothelium.

If endothelium perturbation is not the primary event causing TTP, or exacerbations of the disease, which other etiologic factors contribute to the onset of acute episodes of TTP? We can only speculate about this point. One of the possibilities is that aberrant proteolytic processing of VWF multimers released by endothelial cells through the constitutive pathway, rather than the regulated route, is a factor that contributes to the occurrence of relapses. It is generally thought that acute, regulated release of a Weibel-Palade body-derived storage pool of hyperreactive VWF species, together with impaired proteolytic degradation of these multimers, predisposes to microvascular thrombosis [5,26]. However, this study revealed that regulated secretion of VWF during single episodes or relapses of TTP is not always apparent (as demonstrated by the absence of increase of VWF-propeptide levels during these episodes of the disease). This observation raises the possibility that also impaired proteolytic processing of VWF released through the constitutive pathway, and subsequent accumulation of hyperreactive VWF multimers, contribute to enhanced platelet aggregation in the microvasculature. This view favors the hypothesis that hyperreactive VWF multimers may accumulate slowly in the blood stream, exceeding a threshold level that is required to initiate microvascular platelet aggregation and subsequent exacerbation of the disease [26].

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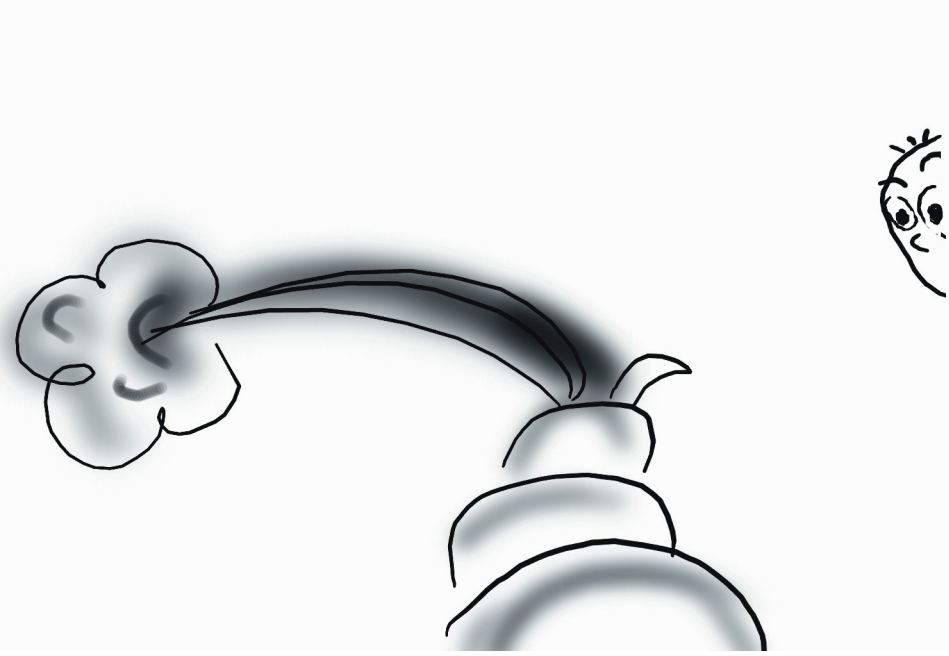
Chapter 7

VON WILLEBRAND FACTOR PROPEPTIDE ENHANCES IL-8-INDUCED NEUTROPHIL ADHESION



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ABSTRACT

In this report we assessed the potential role of the von Willebrand factor (VWF) propeptide as an inflammatory mediator. In a chemotaxis assay, neutrophils were allowed to migrate toward the propeptide, IL-8, or a combination of both. The propeptide alone was not able to induce a chemotactic effect on neutrophils. However, neutrophil adhesion induced by IL-8 increased about 30 % upon co-stimulation with VWF-propeptide ($p < 0.05$). Migration of neutrophils was not significantly affected. Thus, the VWF-propeptide potentiates the chemotactic response of neutrophils toward IL-8. This observation suggests that this VWF-derived protein serves a biological function after its release from the endothelium.

INTRODUCTION

Von Willebrand factor (VWF) is a multimeric plasma glycoprotein that is required for normal haemostasis. VWF is synthesized by endothelial cells and megakaryocytes and originates from its precursor pro-VWF. Endoproteolytic processing of pro-VWF results in mature VWF and the VWF propeptide (also known as VWAg II). In endothelial cells, the propeptide controls polymerization and subsequent targeting of VWF to storage vesicles, the so-called Weibel-Palade bodies. Upon stimulation of endothelial cells, the Weibel-Palade bodies are translocated to the plasma membrane of the cell and mature VWF and its propeptide are co-secreted. After release, mature VWF both controls platelet adhesion and aggregation at sites of vascular injury and acts as a chaperone protein for coagulation factor VIII. In the last few years, increasing evidence has been obtained that the VWF-propeptide might also be involved in a variety of biological processes after it is secreted (reviewed by [1]). It has been suggested that the VWF propeptide may act as an inflammatory mediator. This view stems from the observation that the propeptide is able to promote specific adhesion and spreading of certain leukemia and melanoma cell lines through the integrins $\alpha 4\beta 1$ (VLA4) and $\alpha 9\beta 1$ (VLA9) [2-4]. As both integrins are highly expressed on leukocytes and play a prominent role in transendothelial migration of these cells, it is possible that the propeptide could modulate these processes. To assess the potential role of VWF propeptide as an inflammatory mediator, we have initiated a study on the effect of propeptide on adhesion and migration of neutrophils.

MATERIALS AND METHODS

Purification of VWF-propeptide

Serum-free conditioned medium of CHO cells expressing human VWF-propeptide was kindly provided by dr. U. Schlokat, Baxter Hyland Immuno, Vienna, Austria. The VWF-propeptide was purified from the medium by immuno-affinity chromatography with an immobilized monoclonal antibody (CLB-Pro 35), directed against the propeptide [5]. The purity of VWF-propeptide was established by SDS-PAGE analysis.

Materials

Recombinant human IL-8 was from PreproTech (London, UK), human serum albumin and fibronectin were obtained from our own institute (Sanquin Plasma Products, Amsterdam, The Netherlands).

Isolation of neutrophils

Granulocytes were isolated from blood obtained from healthy volunteers as previously described [6]. Prior to each assay, neutrophils were labeled with 4 mg/ml calcein-AM in Hepes medium for 30 minutes at 37^{o}C. After labeling, the cells were washed twice with and resuspended in Hepes medium [6].

Neutrophil chemotaxis assay

The neutrophil transmigration assay was performed according to [6] except that filters coated, prior to the experiments, for 30 minutes with fibronectin were used instead of endothelial cell monolayers. Migration of neutrophils towards various concentrations of chemoattractants (propeptide, IL-8) was allowed for 5, 15 and 30 minutes. Data are presented as mean ± SEM of 10 experiments and were statistically analyzed with the Student's t-test.

RESULTS AND DISCUSSION

Neutrophils were allowed to migrate through a fibronectin-coated filter toward either IL-8 (1-10 nM), propeptide (0.05-0.2 nM) or a combination of both which were applied in the lower compartment of the microchamber. The percentage of neutrophils that bound to the filter and the percentage transmigrated neutrophils was determined at different time points.

Fig. 1 shows the effect of IL-8 (10 nM) and propeptide (0.1 nM) on neutrophil chemotaxis. As expected, IL-8 clearly induced neutrophil chemokinesis in a typical bell-shape manner. Neutrophils first adhered to the filter after which they migrated

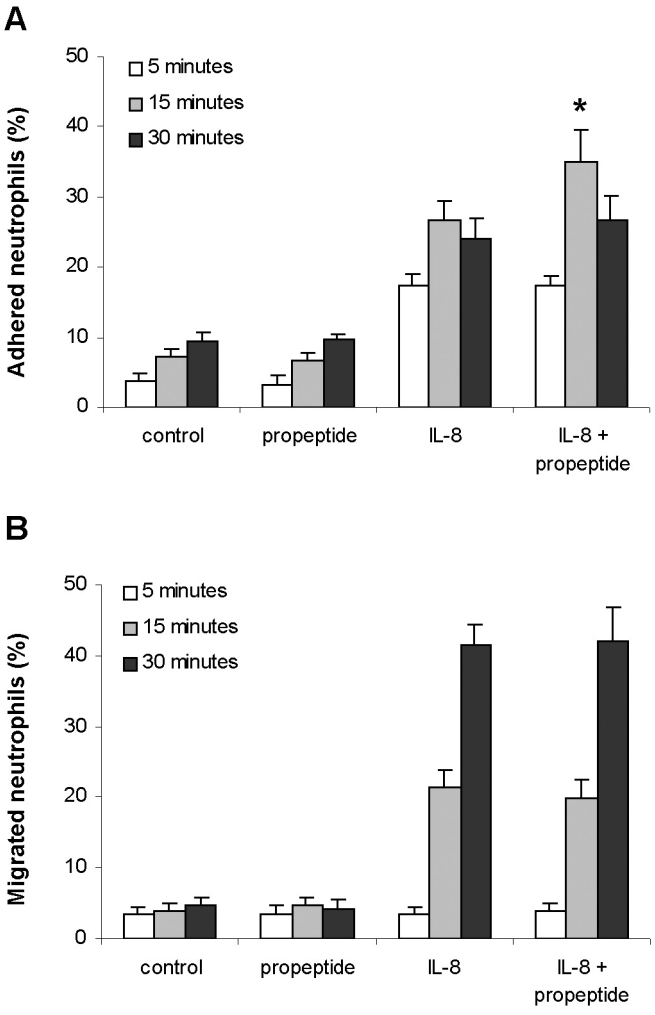

Figure 1. VWF-propeptide enhances the adhesive response of neutrophils toward IL-8. Neutrophils were allowed to migrate through a fibronectin-coated filter toward either IL-8 (10 nM), propeptide (propeptide, 0.1 nM) or a combination of both which were applied in the lower compartment of the microchamber. The percentage of neutrophils that bound to the filter (A) and the percentage transmigrated neutrophils (B) was determined after 5, 15 and 30 minutes of incubation. Data are from 10 experiments and are expressed as mean ± SEM. Significant difference of activity of IL-8 + propeptide from IL-8 is indicated by an asterisk (*, $p < 0.05$).

to the lower compartment. Most neutrophils (26 %) were bound after 15 minutes of incubation with IL-8. After 30 minutes, 41 % of the cells had migrated toward the chemoattractant. Neutrophils did not respond to the propeptide under these conditions. On the other hand, when applied to the lower compartment together with IL-8, the adhesive response of neutrophils toward IL-8 was further enhanced by the propeptide. Neutrophil adhesion induced by IL-8 increased about 35 % upon co-stimulation with VWF-propeptide ($p < 0.05$) after 15 min of incubation. Propeptide decreased IL-8-induced migration. However, as compared to the effect of IL-8 alone, this difference was not significant. Thus, although VWF-propeptide seems unable to induce neutrophil chemotaxis, it potentiates the chemotactic response of neutrophils toward IL-8. This cooperative effect is most prominent at the level of adhesion.

Synergistic interactions between weak and potent chemokines have been previously described. For instance, the weak chemotactic activities of PF-4 and Regakine-1 on leukocytes are enhanced by TNF-α and IL-8, respectively [7,8]. Given its high plasma concentration (5-10 nM, [5])), a feature shared with chemokines such as HCC-1 [9] and also Regakine-1 [8], it seems unlikely that the propeptide serves a role as a primary chemoattractant. The co-targeting of IL-8 and propeptide into a storage compartment of endothelial cells, the Weibel-Palade bodies, and their regulated co-secretion [10,11] rather raises the possibility that the propeptide acts synergistically with IL-8 upon appropriate stimulation of the endothelium, thereby enhancing local adherence of neutrophils.

We can only speculate about the mechanism of this apparent synergistic effect. As the propeptide harbours a recognition site for $\alpha 9\beta 1$ (VLA9, [4]), an integrin involved in neutrophil migration, it is tempting to speculate that interaction of propeptide with this receptor enhances IL-8-mediated adhesion. Similarly, the RGD motif of the propeptide could be involved. It is also possible that VWF-propeptide exerts its synergistic effect through a peptide motif that is structurally closely related to the receptor recognition site of CXC chemokines and the biologically active locus of EMAP II, another potent pro-inflammatory [12]. Further studies are required to get insight into the mechanism of the apparent chemokine-like activity op the propeptide at the molecular basis.

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Chapter 8

GENERAL DISCUSSION

GENERAL DISCUSSION

The aim of the studies described in this thesis was to further unravel the life cycle and fate of Weibel-Palade bodies, the specialized storage vesicles in endothelial cells that contain proteins such as von Willebrand factor (VWF), P-selecting and IL-8. As schematically depicted in Fig. 1, this life cycle can arbitrarily be divided in different phases: a. sorting of proteins into Weibel-Palade bodies, b. intracellular trafficking comprising maturation, transport along microtubules and tethering in the actin network, c. docking, d. exocytosis and, finally, e. endocytosis of released membrane-associated Weibel-Palade body constituents.

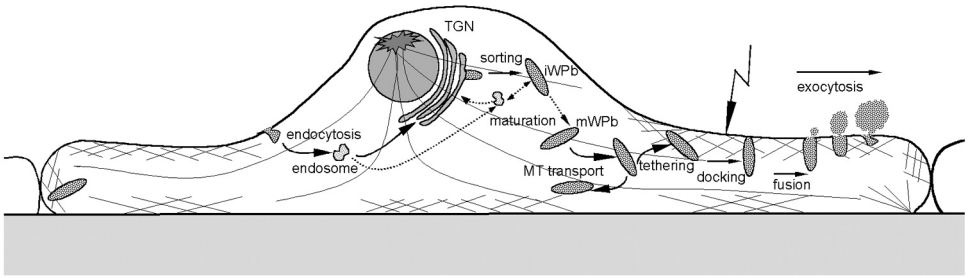

Figure 1. Life cycle of Weibel-Palade bodies. TGN, trans-Golgi Network; iWPb, immature Weibel-Palade body; mWPb, mature Weibel-Palade body; MT transport, microtubule-mediated transport. See text for details.

Sorting of proteins into Weibel-Palade bodies.

The current, widely accepted model for the biogenesis of secretory granules comprises a number of distinct steps occurring at different levels in the secretory pathway (see Fig. 1) [1-4]. These steps include: 1, sorting of granule constituents (e.g. VWF) within the trans-Golgi network (TGN), the so-called “sorting for entry”, 2, budding of constitutive secretory vesicles and immature secretory granules (short-lived vesicular intermediates) from the TGN, and, 3, maturation of the immature secretory granules into mature secretory granules where further refinement of granular constituents occurs, either by “sorting for exit” or “sorting by retention” [1]. In this chapter, protein sorting and maturation processes that are thought to occur in endothelial cell Weibel-Palade bodies will be discussed.

Despite the limited knowledge about the mechanism of protein sorting to Weibel-Palade bodies, evidence is accumulating that VWF not only plays a role in directing the formation of Weibel-Palade bodies, but may also have a helper function in the sorting of other proteins to these secretory vesicles. For instance, trans-

fection of an epithelial cell line which synthesizes P-selectin, but does not retain this receptor in storage vesicles, with VWF-cDNA resulted in the formation of Weibel-Palade body-like organelles, containing both VWF and P-selectin [5]. Similarly, IL-8 is also co-targeted with VWF into Weibel-Palade bodies (Chapter 2). Cells that synthesize IL-8 in substantial amounts, do not store this cytokine in Weibel-Palade bodies, unless VWF and IL-8 are co-expressed. These findings support the view that VWF may trigger sequestration of structurally unrelated proteins into Weibel-Palade bodies. This may also be the case with other Weibel-Palade body constituents, such as CD63, endothelin and α1,3-fucosyltransferase. It is possible that IL-8 and other Weibel-Palade body constituents interact or co-aggregate with VWF in the TGN, a process that may facilitate their retention and condensation within the Weibel-Palade bodies [1]. As VWF is able to bind a variety of proteins and biological compounds [6], it is tempting to speculate that Weibel-Palade body constituents may specifically interact with VWF preceding maturation of secretory vesicles. This view is supported by our finding that IL-8 specifically binds with VWF in vitro under conditions that may mimic the TGN milieu (Romani de Wit, unpublished data). Similarly, coexpression studies of VWF and factor VIII (a coagulation factor that readily binds to VWF) in AtT-20 cells and vascular endothelial cells, showed co-localization of factor VIII and VWF in storage vesicles [7]. Apparently, VWF-mediated sorting of factor VIII was due to interaction of these proteins in the TGN.

So far, no distinction has been made between the role of mature VWF in protein sorting and that of its propeptide. However, the propeptide moiety of VWF may also play an important role in the targeting process (see below). Two models of protein sorting that are not mutually exclusive have been reported: the multimerization hypothesis and the propeptide targeting model. The multimerization hypothesis advocates that storage of VWF is directed by selective aggregation (i.e. multimerization) and condensation [5,8,9], a process that underlies the sorting and targeting of many other proteins and hormones destined for regulated secretion (reviewed in [1,10,11]). The propeptide hypothesis considers that the VWF-propeptide contains a targeting signal that interacts with a carrier protein or receptor in the Golgi membrane to mediate targeting of VWF (and the propeptide itself) to storage [12]. According to the latter view, VWF would traffic to storage vesicles only through its noncovalent interaction with its propeptide. A recent report showed that the VWF-propeptide is also capable of targeting a protein structurally unrelated to VWF, the complement protein C3, into storage vesicles, provided that VWF-propeptide is covalently linked to this foreign protein [13]. It is very well possible that either VWF or its propeptide interact noncovalently with other Weibel-Palade body residents to sequester them into the storage vesicles.

Intracellular trafficking of Weibel-Palade bodies

Once (premature) Weibel-Palade bodies are formed and are packed with a variety of bioactive molecules, what is the fate of these vesicles preceding exocytosis? Based on the data presented in this thesis (chapters 3 and 4) and on the findings reported elsewhere, intracellular trafficking of Weibel-Palade bodies is likely to comprise a succession of distinct processes.

1. Maturation of Weibel-Palade bodies

Little is known about the maturation of Weibel-Palade bodies; not even whether these vesicles undergo maturation at all. In general, organelles involved in the regulated secretory pathway of a variety of cell types are initially formed in the TGN as immature secretory granules. They undergo maturation and become mature secretory granules. This maturation involves selective removal of molecules that are not destined for secretion. For example, membrane proteins such as mannose 6-phosphate receptor, TGN endoprotease furin and carboxypeptidase D, are removed from immature vesicles in a clathrin-dependent process and end up in endosomes [14] [15] [16]. Furin and carboxypeptidase D not only exert their enzymatic activities in the TGN, but are also active in immature secretory granules [17]. Interestingly, furin has also been shown to cleave pro-VWF into mature VWF and VWF-propeptide. This process is thought to take place in the TGN [18-21]. Although the process of maturation of Weibel-Palade bodies has not yet been documented, it is possible that proteolytic cleavage of pro-VWF occurs in immature Weibel-Palade bodies. Co-localization studies of furin with VWF containing vesicles could provide more insight in the maturation process of these vesicles.

In this thesis, indirect evidence was provided that Weibel-Palade bodies indeed undergo maturation. In chapter 3 we describe the existence of different pools of Weibel-Palade bodies. Some VWF containing vesicles barely move as if they were tethered, others seem to travel in a stochastic manner and frequently reverse their direction. Furthermore, all Weibel-Palade bodies moved with remarkably low speed, not exceeding 10 nm/s. Previous studies on other cell types have shown that secretory vesicles travel in different motion. Fast, microtubule-associated vesicles, travelling from the Golgi to the plasma membrane, move at an average speed of about 1 μm/s, and are thought to represent a subset of immature vesicles [22-25]. Upon arrival at the plasma membrane, vesicles are tethered which decreases considerably their motility to about 50 nm/s [25,26]. Mature vesicles are trapped in the dense meshwork of the actin cortex that prevents movement and are awaiting release [25,27]. The real time data from chapter 3 indicate that tethered Weibel-Palade bodies are trapped in the actin cortex of endothelial cells. On the other hand, those Weibel-Palade bodies that move in all directions are reminiscent of microtu-

bule-associated granules [22]. Based on their behavior and dynamics, it is tempting to speculate that tethered, slowly moving WPbs represent mature vesicles, whereas the other pool consists of immature microtubule-associated vesicles. Whether this is indeed the case remains to be established.

2. Weibel-Palade body transport along microtubules

It is now well established that vesicle trafficking proceeds both on microtubules and on microfilaments and involves cytoskeletal-associated motor proteins [28-30]. Microtubules are thought to provide tracks for movement over long distances while actin filaments preferentially control movement to local sites [31]. Vesicles have motor proteins for both actin- and microtubule-mediated transport. Also a direct interaction between actin- and microtubule-dependent motors themselves has been found, suggesting the existence of an extensive cross-talk between the two cytoskeletal systems. Similarly, a role of microtubules in Weibel-Palade body trafficking has been demonstrated. In fact, intact microtubules are essential for correct processing and regulated secretion of VWF by endothelial cells [32]. Disruption of microtubules inhibited thrombin- and histamine-induced VWF secretion [32,33]. In this thesis (chapter 3), additional evidence was provided that microtubules play an important role in the intracellular transport of Weibel-Palade body. The stochastic movement of Weibel-Palade bodies observed in resting endothelial cells is reminiscent of microtubule-associated trafficking. When endothelial cells were stimulated with cAMP-raising agonists, Weibel-Palade bodies migrated perinuclearly (chapter 3). They were specifically recruited to the microtubule organizing centre as schematically illustrated in Figure 1. Based on their location and dynamics, this process most likely involves active transport of Weibel-Palade bodies along microtubules. Intracellular vesicles are transported along microtubules by utilising microtubule-associated motor proteins such as kinesin and dynein [34-36]. Kinesin moves towards the fast growing (or plus end), away from the microtubule organizing centre, and cytoplasmic dynein moves towards the minus end [30,37]. The movement of Weibel-Palade bodies in a zigzag fashion, in both plus- as minus-end direction, suggests that both types of motor proteins are involved in Weibel-Palade body trafficking in resting cells. The mechanisms underlying Weibel-Palade body trafficking along the cytoskeletal tracks have not been identified so far. A growing number of interactions between small GTPases and kinesin-like proteins has been described in literature. For instance, rab6 directly binds rabkinesin6 [38], Rho, Rac and Cdc42 have been found to interact with the kinesin-associated protein kinectin [39]. Since such molecules have also been identified in endothelial cells [40-42], it is tempting to speculate that GTPases-kinesin interactions may contribute to Weibel-Palade body-trafficking. Future studies could reveal whether these proteins

commonly involved in intracellular trafficking are also associated with Weibel-Palade bodies in endothelial cells.

3. Tethering of Weibel-Palade bodies in the actin cortex

Upon arrival at the plasma membrane, secretory vesicles are tethered which decreases considerably their motility [25,26]. These vesicles are sequestered in the dense meshwork of the actin cortex that forms a physical barrier to exocytosis for the majority of secretory granules in resting cells [43-45]. Thus, tethering is an important step in the trafficking of vesicles and is essential for exocytosis. Although the co-localization of Weibel-Palade bodies and the actin cortex was not studied, a number of Weibel-Palade bodies seemed indeed to be trapped near the plasma membrane (chapter 3). Apparently, Weibel-Palade bodies are recruited at the cell periphery awaiting the trigger for release. The process of tethering is regulated by a complex series of protein interactions, most of which remain to be identified in endothelial cells.

Recent data showed co-localization of Weibel-Palade bodies with another small GTP-binding protein, Rab27 [46]. This GTPase regulates a variety of processes involving melanosome motility in melanocytes [47], lytic granule release in cytotoxic lymphocytes [48], and exocytosis of dense core granules in platelets [49,50] and pancreatic β-cells [51]. The tethering mechanism of melanosomes has been investigated in more detail. It seems that Rab27 recruits myosin Va to the melanosome and that myosin Va then tethers the melanosomes to subcortical actin filaments at the periphery of the cell [52]. Weibel-Palade bodies may be recruited to the plasma membrane by a similar trapping mechanism. It would be very interesting to elucidate the involvement of these different GTPases in Weibel-Palade body.

Docking of Weibel-Palade bodies with the plasma membrane

Activation of cells induce rearrangements in the actin cytoskeleton occur, which enable docking of vesicles at exocytic sites. Docking of exocytic vesicles with the plasma membrane has been shown to involve a protein complex known as the “exocyst”. The exocyst localizes specifically to regions of active secretion. Recent data have demonstrated that many small GTPases regulate the exocyst, including members of the Rab [53], Rho [54-56] and Ral families [57-60]. Interaction of Rab, Rho and Cdc42 with different exocyst components has only been identified in yeast. The mammalian exocyst (or Sec6/8 complex) associates with activated RalA through the binding to the exocyst subunit Sec5, leading to the targeting of synaptic vesicles to the plasma membrane. Furthermore, RalA has been implicated in the regulation of processes such as endocytosis and actin cytoskeleton dynamics. It is likely that the functional role of the majority of small GTP-binding proteins in endo-

thelial cells is similar, if not identical, to that defined in other cell types. In endothelial cells, RalA associates with Weibel-Palade bodies [61] and in chapter 4, evidence is provided that RalA is involved in the exocytosis of these vesicles. Therefore, it is tempting to speculate that docking of Weibel-Palade bodies occurs through interaction of Weibel-palade body-associated RalA and a putative exocyst at the plasma membrane. Studies on the presence of components of the exocyst complex in endothelial cells and their functional involvement in exocytosis of Weibel-Palade bodies are certainly worth further investigations.

Exocytosis

Exocytosis of Weibel-Palade bodies occurs upon stimulation of endothelial cells, either with Ca^{2+}- or cAMP-raising agents. Contrary to previous suggestions, our data indicate that Weibel-Palade bodies do not fuse with each other during exocytosis. Chapter 3 demonstrates that exocytosis of Weibel-Palade bodies comprises fusion of individual vesicles with the plasma membrane, gradual release and diffusion of Weibel-Palade body contents into the extracellular compartment. The mechanism underlying fusion between Weibel-Palade bodies and the plasma membrane remains to be established.

During exocytosis, the different residents of Weibel-Palade bodies are either secreted (e.g. VWF, propeptide, IL-8) or exposed at the cell surface (P-selectin and CD63). Striking is the relative long life span of Weibel-Palade body exocytosis (minutes, chapter 3) compared to that of other secretory vesicles (seconds) [24,26]. The apparent slow exocytosis of Weibel-Palade bodies could be due to the compact, crystalloid structure of VWF, which may hamper its release and dissolution. The possible interaction of VWF with other Weibel-Palade body constituents or with proteins localized at the plasma membrane may also affect the rate of exocytosis.

Slow exocytosis may be of important physiological significance. As a result of slow exocytosis, stimulated endothelial cells may present at their surface focal sites with a high concentration of VWF, which, together with other Weibel-Palade body constituents, could serve a role in adequately recruiting distinct plasma proteins, blood cells or matrix components at sites of vascular injury. Pertinent to this point is the observation of André and co-workers [62] who showed that platelets adhered rapidly, though transiently, to VWF secreted at the luminal face of endothelial cells upon triggering of the cell with Ca^{2+}-agonists. The observation that adherence of platelets to the endothelium was transient (minutes) corresponds with the time course of exocytosis. In addition, focal sites expressing high levels of P-selectin and IL-8 may contribute to the rapid recruitment of leukocytes by endothelial cells after simulation. Pertinent to this view is the observation that in VWF-deficient mice, [63] Weibel-Palade body formation is defective resulting in reduced P-selectin

expression at the cell surface. This, in turn, hampers neutrophil recruitment in early phases of inflammation. Interestingly, the time frame of P-selectin-mediated rolling of leukocytes on the endothelium [64] is in the same order of magnitude as the exocytosis process and concomitant patch formation described in chapter 3. Based on our model of focal site formation, also a defect in IL-8 secretion could impair leukocyte recruitment found in VWF-deficient mice. Interestingly, the results described in chapter 7 show that the VWF-propeptide acts synergistically with IL-8 by enhancing the adhesion of neutrophils. The co-localization of IL-8 and propeptide in Weibel-Palade bodies and their co-secretion may favor cooperative effects. Thus, focal sites expressing these two proteins at high levels could also contribute to the rapid recruitment of neutrophils to endothelial cells after stimulation. Although not studied in this thesis, the propeptide may also be involved in monocyte adhesion to the endothelium. Actually, monocytes can adhere to the endothelium by various integrins, such as $\alpha 4\beta 1$, on their surfaces. Since the VWF-propeptide is a ligand for $\alpha 4\beta 1$, it is possible that the propeptide may also participate in the recruitment of monocytes by the endothelium. Pertinent to this point are the observations of Methia et al [65] that in VWF-deficient mice, atherosclerotic lesion formation was reduced. Localized reduction of artherosclerosis correlated with a reduced recruitment of monocytes.

Taken together, ample evidence is provided that emphasizes the importance of Weibel-Palade body exocytosis at the interface between inflammatory and the hemostatic response. Furthermore, the biological functions of the different Weibel-Palade body constituents seem more intertwined than previously anticipated. It would be fascinating to determine in real-time studies whether blood components interact indeed with Weibel-Palade body constituents at these specific focal sites during vascular perturbation.

Endocytosis

Although not studied in this thesis, endocytosis can also be considered as a step in the life cycle of Weibel-Palade bodies (see figure 1). Endocytosis does not lead to the formation of new Weibel-Palade bodies, but produces small vesicles that end up in for example endosomes. Endocytosis enables the recycling of membrane-bound Weibel-Palade body constituents from the plasma membrane back to the TGN, lysosomal compartment or even to new Weibel-Palade bodies. Indeed, after translocation on the plasma membrane following exocytosis, the trans-membrane proteins P-selectin and CD63 are rapidly internalized [66-68]. P-selectin recycles to the Weibel-Palade bodies via the TGN. Unlike P-selectin, endocytosed CD63 accumulates in endosomes and either recycles directly to Weibel-Palade bodies or is targeted to lysosomes. Besides the different routes used by P-selectin and CD63

to re-enter Weibel-Palade bodies, also the kinetics and extent of recycling are very different. Recycling of proteins and the fusion of endosomes with Weibel-Palade bodies may provide a new supply of elements required to support the life cycle of Weibel-Palade bodies. This new supply may, for example, be useful for tethering of Weibel-Palade bodies to the plasma membrane. We hypothesize that recycling of proteins to Weibel-Palade bodies could be involved in the maturation process of these vesicles. Future research should reveal whether this is indeed the case.

We can only speculate about the mechanisms involved in endocytosis. Evidence has been provided of the implication of the small GTPase RalA in endocytosis of EGF and insulin receptors [69]. GTP hydrolysis was shown to be critical for RalA endocytosis. Similarly, RalA, which is associated with Weibel-Palade bodies, may not only play a role in the exocytosis of Weibel-Palade bodies (chapter 4) but may also be involved in the endocytotic process. No data are currently available with respect to the involvement of RalA in endocytosis of Weibel-Palade body components. It is tempting to speculate that RalA modulates re-entry of endocytosed CD63 and P-selectin into newly formed, immature Weibel-Palade bodies.

Life span of a Weibel-Palade body?

The life cycle of Weibel-Palade bodies is affected by distinct physiological conditions. During steady-state conditions, the basal turnover of Weibel-Palade bodies is estimated to be about 24 h [68]. Indeed, Weibel-Palade bodies undergo low-level spontaneous exocytosis even in the absence of stimulation [70]. Upon stimulation, endothelial cells undergo different secretory processes depending on the agonists to which they are exposed. These processes are of importance for the release of Weibel-Palade bodies and consequently for their life span. Based on our knowledge and observations described in this thesis, we propose a model that describes the processes occurring during stimulation with either Ca^{2+}- or cAMP raising agents (Figure 2). These two stimulation processes could be considered to represent physiological (cAMP-raising agonists) and pathophysiological (Ca^{2+}-raising agonists) mechanisms (also reviewed in [71]).

As illustrated in Figure 2, Ca^{2+}-raising agonists, such as thrombin or histamine, induce cell retraction very rapidly (within 5 minutes) producing gaps between cells. This is accompanied by redistribution of actin and myosin into stress fibers [33]. Exocytosis of Weibel-Palade bodies is observed also within a few minutes after stimulation (chapter 3). In vivo, Ca^{2+}-raising agonists are mediators of inflammation or thrombosis, usually acting locally. Cell retraction possibly results in edema formation or exposure of the subendothelium (and subsequent thrombotic events). Fast release of inflammatory and pro-thrombotic agents, such as those present in Weibel-Palade bodies, may amplify these pathophysiological processes. Ca^{2+}-me-

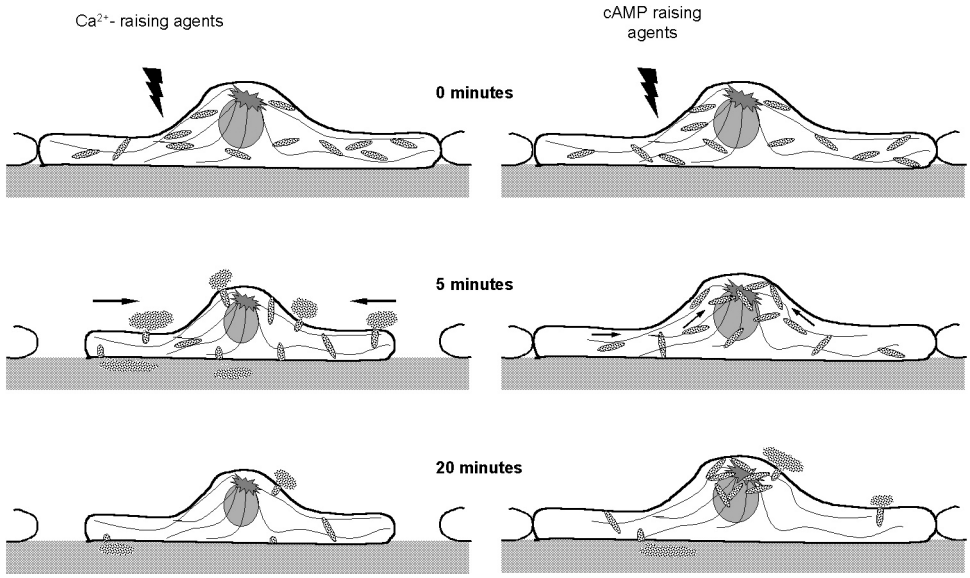

Figure 2. Dynamics of Weibel-Palade bodies upon stimulation with Ca2+- and cAMP-raising agonists. Note the different effects of these agonists on the cell-shape, Weibel-Palade recruitment and the start of Weibel-Palade body exocytosis. See text for details.

diated secretion, e.g. in response to vascular damage, most likely reflects mobilization of the entire WPb population to accomplish adequate release of bioactive molecules at sites of vascular injury. Consequently the life span of Weibel-Palade bodies under pathophysiological conditions is reduced.

cAMP-raising agonists, e.g. epinephrin and forskolin, induce disruption of stress fibers, strengthening of the cortical actin rim, and tightening of cell-cell contacts [33]. Following such stimulus, Weibel-Palade bodies are recruited perinuclearly, and more specifically move toward the microtubular organizing center. Perinuclear recruitment most likely involves trafficking along microtubules and association of microtubule-associated motor proteins. cAMP-induced exocytosis starts about 20 minutes after stimulation, a process that is delayed compared to that induced by Ca^{2+}-raising agonists. Interestingly, after addition of cAMP-raising agonists less Weibel-Palade bodies seem to undergo exocytosis compared to Ca^{2+}-raising agents. Apparently, the cAMP-dependent perinuclear recruitment of Weibel-Palade bodies may be a mechanism to retrieve the storage pool of pro-thrombotic and pro-inflammatory mediators from the plasma membrane and prevents excessive release of their contents. As such, it may provide a means to limit excessive release in Weibel-Palade bodies under physiological conditions, such as physical exercise or other stress situations [71]. Under physiological conditions, two pools

of Weibel-Palade bodies may exist: One pool, that is probably already tethered near the plasma membrane, undergoes exocytosis and has a short life span. The second (probably immature) pool of Weibel-Palade bodies escapes exocytosis, perhaps because it is not tethered, resulting in a longer life span.

The existence of “physiological” or “pathophysiological” secretion of Weibel-Palade bodies is not always obvious in pathophysiological conditions such as thrombotic thrombocytopenic purpura (TTP, chapter 6). It is generally thought that acute, regulated release of a Weibel-Palade body-derived storage pool of hyperreactive VWF species, together with impaired proteolytic degradation of these multimers, predisposes to microvascular thrombosis [72]. We can’t exclude, however, that slow accumulation of hyperreactive VWF multimers in the bloodstream is a result of “physiological”, cAMP-mediated exocytosis exceeding threshold levels required to initiate microvascular platelet aggregation and subsequent exacerbation of the disease.

In conclusion, this thesis has brought forward different issues concerning the life cycle of Weibel-Palade bodies. This life cycle comprises multiple processes and mechanisms of remarkable complexity. We and other studies have provided evidence that the different steps in this life cycle most likely are of important physiological significance in the processes underlying hemostatic, thrombotic and inflammatory responses. However, the different phases and stages of the life cycle of Weibel-Palade bodies are far of being unraveled and merit further investigation.

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SUMMARY

Vascular endothelial cells are equipped with a machinery that, upon perturbation, allows prompt delivery of a number of bioactive substances, including hormones, receptors and adhesive molecules, to the surface of the cell. A distinct subset of proteins destined to be released upon stimulation of the endothelium stems from Weibel-Palade bodies, typical and morphologically organized storage vesicles that release their contents by regulated exocytosis. Weibel-Palade bodies are able to store a variety of proteins with different biological functions such as the multimeric glycoprotein von Willebrand factor (VWF) that is required for normal haemostasis, the leukocyte adhesion receptor P-selectin, and the chemokine interleukin-8 (IL-8). Correct delivery of these proteins to the cell membrane or to the circulation is essential for the biological role of these proteins. Indeed, regulated secretion of VWF provides an adequate means for endothelial cells in controlling the arrest of bleeding upon vascular damage. In addition, effective translocation of P-selectin from Weibel-Palade bodies to the cell surface is critical for the binding and rolling of leukocytes on the endothelium at sites of inflammation. Similarly, regulated exocytosis of IL-8 provides an effective means of controlling local leukocyte extravasation. Taken together, regulated exocytosis of Weibel-Palade bodies serves several physiological functions including inflammatory and haemostatic responses. However, the mechanisms such as sorting of proteins to Weibel-Palade bodies, Weibel-Palade body trafficking in the cell and secretion of their content into the circulation, are far of being understood. In this thesis, I tried to get more insight into the life cycle of Weibel-Palade bodies and the extracellular function of one of their constituents.

Chapter 1 provides background information about this subject and describes the biosynthesis, processing and secretion of VWF, the major constituent of Weibel-Palade bodies, known so far. It introduces the importance of protein storage in Weibel-Palade bodies which forms the basis of research on the life cycle of these vesicles described in the following three chapters.

The first step of the life cycle of Weibel-Palade bodies is the sorting process of proteins into these storage organelles. Despite the limited knowledge about the mechanism of protein sorting to Weibel-Palade bodies, evidence is accumulating that VWF has a helper function in the sorting of other proteins (e.g. P-selectin) to these secretory vesicles. In **Chapter 2**, the role of VWF in the sorting of IL-8 into Weibel-Palade bodies was investigated. For this purpose, an endothelial cell line that had retained the typical phenotypic characteristics of primary endothelial cells, but that has lost the ability to produce VWF in appreciable amounts was used. Strikingly, this cell line is no longer able to store IL-8 in Weibel-Palade bodies. By transducing this cell line with a vector encoding a VWF-green fluorescent protein chimera (VWF-GFP), we restored the expression of VWF and the generation of Weibel-Palade body-like organelles. VWF-GFP-expressing cells also showed

significant co-localization of VWF-GFP and IL-8 in these storage vesicles. These observations indicated that VWF plays an active role in sequestering IL-8 into Weibel-Palade bodies.

After sorting into Weibel-Palade bodies, proteins such as VWF are transported throughout the cell and are secreted upon stimulation of endothelial cells. **Chapter 3** describes possible mechanisms involved in the intracellular trafficking of Weibel-Palade bodies and their exocytosis. For that purpose, the above-mentioned VWF-GFP chimera to infect wild-type endothelial cells was used. This approach allowed direct visualization of the routing and fate of Weibel-Palade bodies upon stimulation in live cells. Different pools of Weibel-Palade bodies were observed: some vesicles barely moved as if they were tethered, others seemed to travel in a stochastic manner and frequently reverse their direction. It was hypothesized that Weibel-Palade bodies undergo maturation during transport along microtubules. Upon arrival at the plasma membrane, tethered (mature?) Weibel-Palade bodies are trapped in the actin cortex of endothelial cells that forms a physical barrier to exocytosis. Apparently, Weibel-Palade bodies are recruited at the cell periphery awaiting a trigger for release. Exocytosis of Weibel-Palade bodies occurs upon stimulation of endothelial cells, either with Ca^{2+}- or cAMP-raising agonists. These results suggested that exocytosis of Weibel-Palade bodies comprises fusion of individual vesicles with the plasma membrane, gradual release and diffusion of Weibel-Palade body content into the extracellular compartment. As a result of slow exocytosis, endothelial cells may present at their surface focal sites with high concentrations VWF, IL-8 and P-selectin which, in turn, may play a role in focal adhesion of blood constituents to the endothelium upon vascular injury. In addition to exocytosis, cAMP-raising agonists induce clustering of a subset of Weibel-Palade bodies in the perinuclear region of the cell. The cAMP-dependent recruitment of Weibel-Palade bodies may be a mechanism to retrieve the storage pool of pro-thrombotic and pro-inflammatory mediators from the plasma membrane and to prevent excessive exocytosis of Weibel-Palade bodies.

Chapter 4 focuses on the role of RalA, a small GTP-binding protein shown to be associated with Weibel-Palade bodies, in the regulated exocytosis of these vesicles. Activation of endothelial cells by thrombin resulted in transient cycling of RalA from its inactive GDP- to its active GTP-bound state, a process which coincided with release of VWF. Furthermore, expression of constitutively active Ral in endothelial cells resulted in exocytosis of Weibel-Palade bodies whereas expression of a dominant negative Ral variant did not show this effect. Taken together, RalA seems to be involved in regulated exocytosis of Weibel-Palade bodies from endothelial cells. RalA associates with the exocyst of different cell types to target vesicles to the plasma membrane. It is tempting to speculate that also in endothelial

cells, Weibel-Palade body-associated RalA interacts with a putative exocyst leading to docking of Weibel-Palade bodies at the plasma membrane. RalA may also be involved in the endocytosis of integral membrane proteins like P-selectin and CD63 that are contained within Weibel-Palade bodies.

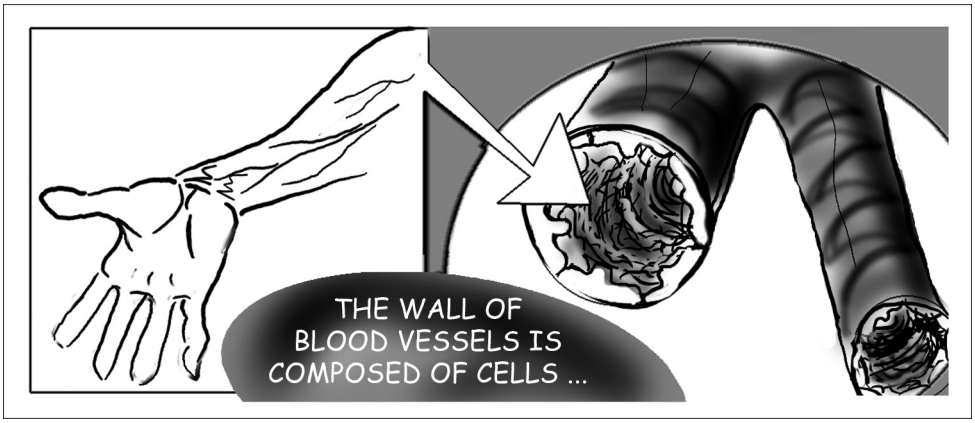
As mentioned above, Weibel-Palade bodies store a variety of proteins with different biological functions and secrete them into the circulation upon stimulation of endothelial cells. There is no doubt that regulated exocytosis of Weibel-Palade bodies serves several physiological functions including inflammatory and haemostatic responses. The second part of this thesis focuses on the biological significance of the VWF-propeptide, one of the Weibel-Palade body constituents. **Chapter 5** serves as introduction of this part and illustrates the potential role of the propeptide as a marker of endothelial cell perturbation. Measurement of both VWF and VWF-propeptide levels allows discrimination between acute and chronic endothelial perturbation. Further, this chapter shows that the biological function of the VWF-propeptide is more diverse than previously anticipated. This is starting point for the following chapters.

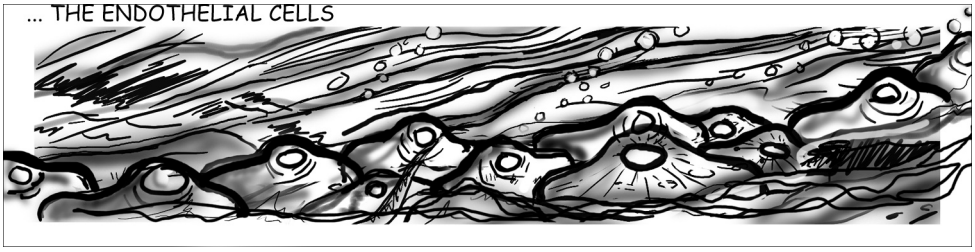
VWF-propeptide was used as a marker for endothelial cell perturbation in **Chapter 6** to determine whether endothelial cell activation is the primary event in the pathogenesis of thrombotic thrombocytopenic purpura (TTP). The degree of endothelial cell involvement was assessed in TTP patients at admission, exacerbation and remission by measurement of VWF and VWF-propeptide levels. Elevated levels of both VWF and its propeptide were found at admission. These levels decreased immediately upon plasma exchange therapy. However, plasma VWF and propeptide concentrations did not change, even at exacerbations. We conclude that endothelial cell activation is not the primary event leading to TTP. Vascular perturbation seems a consequence rather than a cause of the disease.

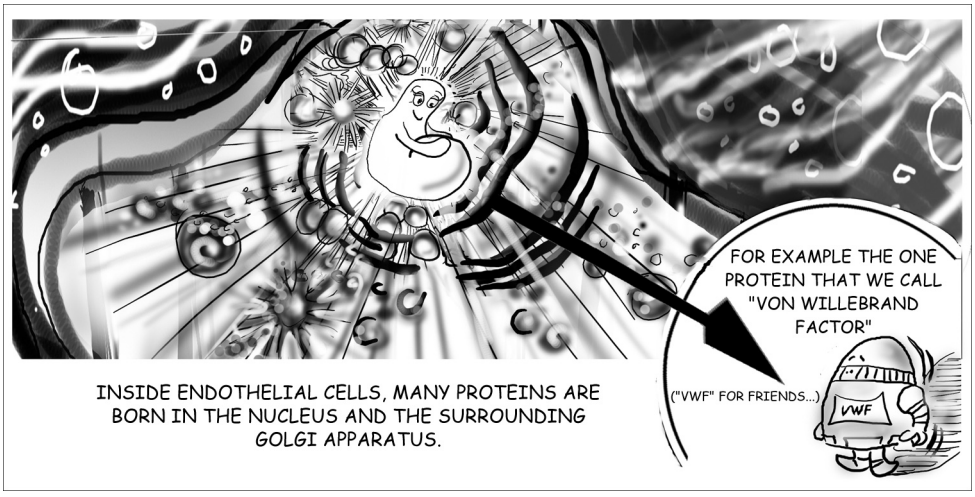
The VWF-propeptide is able to promote specific adhesion and spreading of certain leukemia and melanoma cells through the integrins α4ß1 (VLA4) and α9ß1 (VLA9). As both integrins are highly expressed on leukocytes and play a prominent role in transendothelial migration of these cells, we hypothesized that the VWF-propeptide could modulate this process. In **Chapter 7** the potential role of the VWF-propeptide as an inflammatory mediator was investigated. In a chemotaxis assay, neutrophils were allowed to migrate toward the propeptide, IL-8, or mixtures of both. The propeptide alone had no chemotactic effect on neutrophils. However, neutrophil adhesion induced by IL-8 increased significantly upon co-stimulation with VWF-propeptide. The co-storage of IL-8 and propeptide in Weibel-Palade bodies and their co-secretion raises the possibility that the propeptide acts synergistically with IL-8 upon appropriate stimulation of the endothelium, thereby enhancing local adherence of neutrophils.

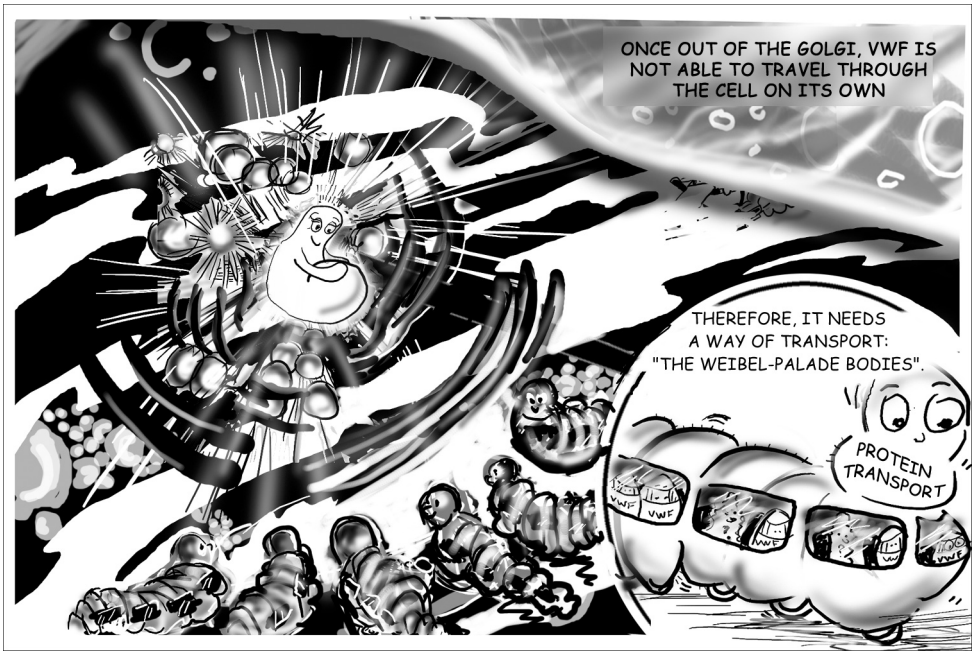
In conclusion, this thesis has brought forward different issues concerning the life cycle of Weibel-Palade bodies. This life cycle comprises multiple processes and mechanisms of remarkable complexity, many of which remain to be unraveled. Evidence has been provided that, besides its important role inside the cell, the Weibel-Palade body resident VWF-propeptide may also have a biologically significant role after its release into the circulation. Taken together, biogenesis and exocytosis of Weibel-Palade bodies are essential in the processes underlying hemostatic, thrombotic and inflammatory responses.

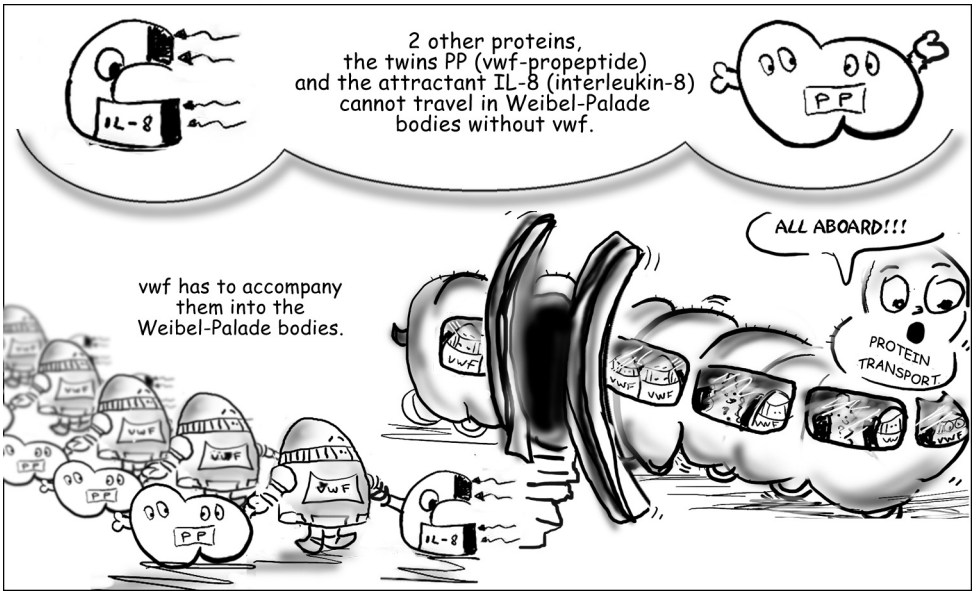
LAYMAN SUMMARY

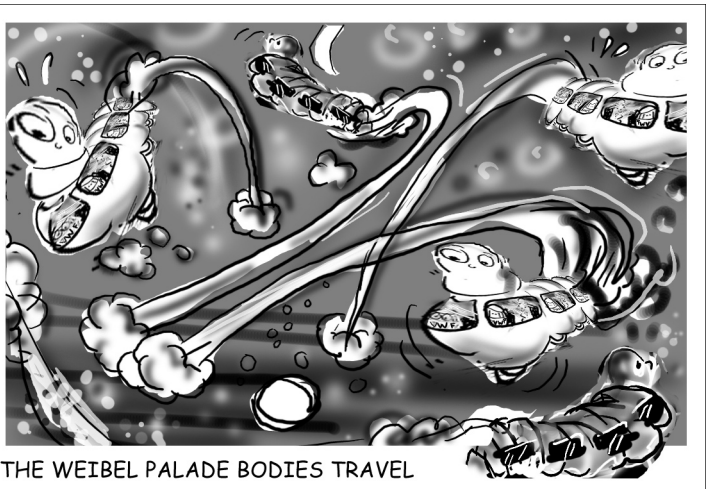

THE WALL OF
BLOOD VESSELS IS
COMPOSED OF CELLS ...


... THE ENDOTHELIAL CELLS

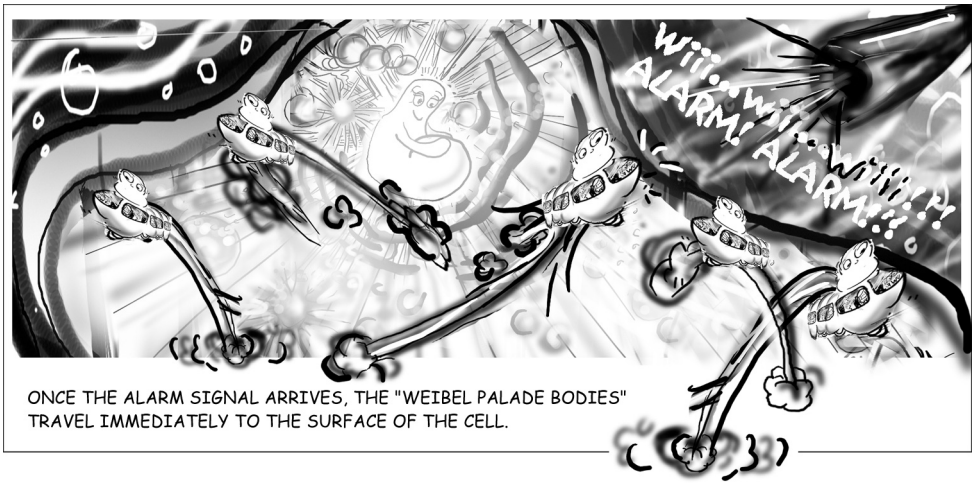

INSIDE ENDOTHELIAL CELLS, MANY PROTEINS ARE
BORN IN THE NUCLEUS AND THE SURROUNDING
GOLGI APPARATUS.
FOR EXAMPLE THE ONE
PROTEIN THAT WE CALL
"VON WILLEBRAND
FACTOR"
("VWF" FOR FRIENDS...)
VWF

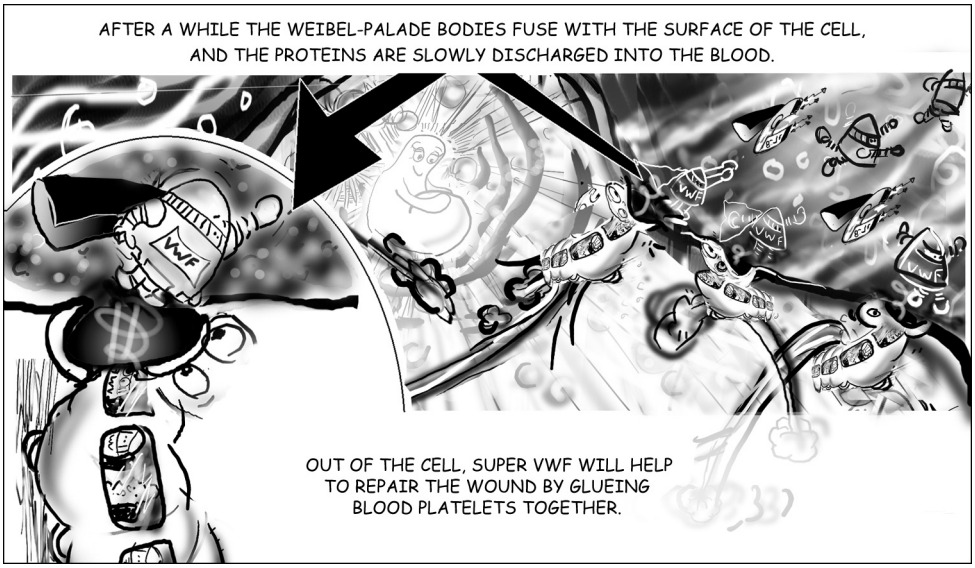

ONCE OUT OF THE GOLGI, VWF IS NOT ABLE TO TRAVEL THROUGH THE CELL ON ITS OWN
THEREFORE, IT NEEDS A WAY OF TRANSPORT: "THE WEIBEL-PALADE BODIES".
PROTEIN TRANSPORT
VWF

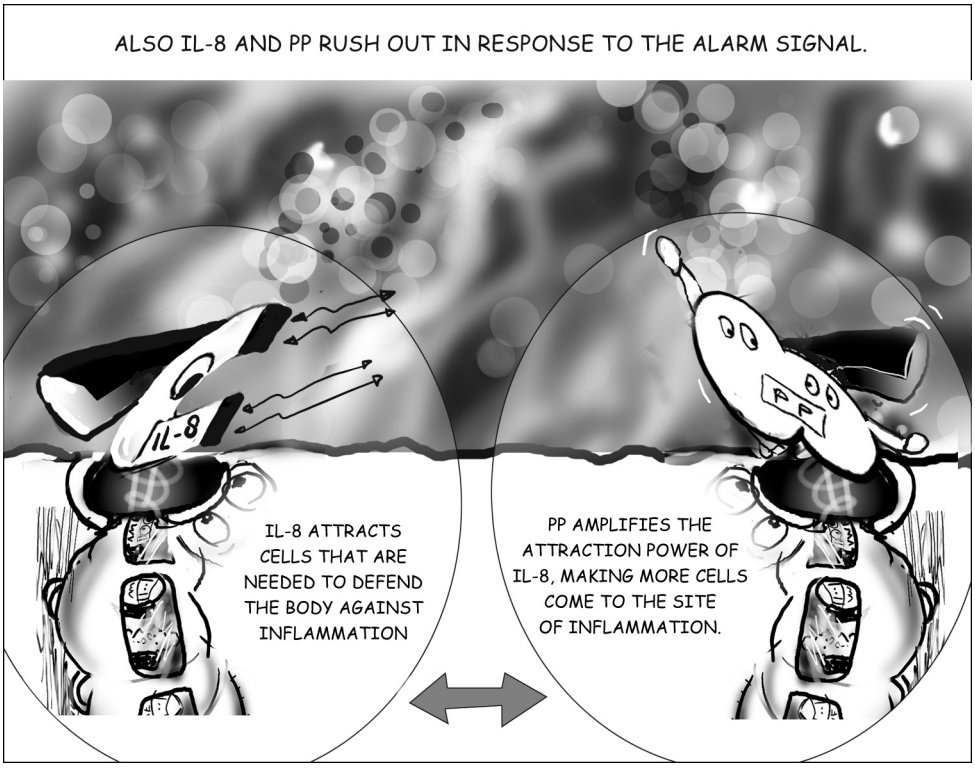

IL-8
2 other proteins, the twins PP (vwf-propeptide) and the attractant IL-8 (interleukin-8) cannot travel in Weibel-Palade bodies without vwf.
PP
ALL ABOARD!!!
vwf has to accompany them into the Weibel-Palade bodies.
PROTEIN TRANSPORT
VWF

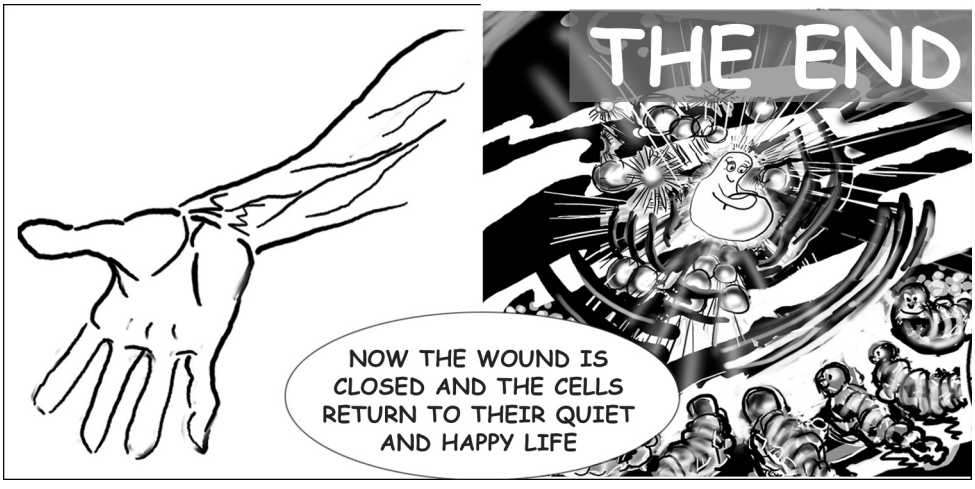


THE WEIBEL PALADE BODIES TRAVEL THROUGH THE CELL IN ALL DIRECTIONS AS LONG AS THERE IS NO EMERGENCY SIGNAL FROM THE BODY.


WIII...WIII...WIIII!?!
ALARM! ALARM!?!
ONCE THE ALARM SIGNAL ARRIVES, THE "WEIBEL PALADE BODIES"
TRAVEL IMMEDIATELY TO THE SURFACE OF THE CELL.


AFTER A WHILE THE WEIBEL-PALADE BODIES FUSE WITH THE SURFACE OF THE CELL,
AND THE PROTEINS ARE SLOWLY DISCHARGED INTO THE BLOOD.
VWF
OUT OF THE CELL, SUPER VWF WILL HELP
TO REPAIR THE WOUND BY GLUEING
BLOOD PLATELETS TOGETHER.


ALSO IL-8 AND PP RUSH OUT IN RESPONSE TO THE ALARM SIGNAL.
IL-8
IL-8 ATTRACTS CELLS THAT ARE NEEDED TO DEFEND THE BODY AGAINST INFLAMMATION
PP
PP AMPLIFIES THE ATTRACTION POWER OF IL-8, MAKING MORE CELLS COME TO THE SITE OF INFLAMMATION.


THE END
NOW THE WOUND IS CLOSED AND THE CELLS RETURN TO THEIR QUIET AND HAPPY LIFE

SAMENVATTING
VOOR NIET-INGEWIJDEN

Wanneer een persoon zich verwondt en een bloeding optreedt zal normaal gesproken snel de bloedstolling beginnen en zich een korstje vormen dat de wond afdekt en het begin is van genezing. Een van de belangrijkste factoren die betrokken is bij dit bloedstollingsproces is een eiwit, genaamd *'von Willebrand factor'*, dat gemaakt en opgeslagen wordt in de zogenaamde endotheel cellen die de wand van bloedvaten bekleden. Als deze endotheel cellen worden geprikkeld, dan pas wordt de von Willebrand factor uitgescheiden in de bloedbaan om zijn werk te doen. Endotheel cellen kunnen op twee manieren geprikkeld worden: 1) door het ontstaan van een beschadiging in het bloedvat, bijvoorbeeld door een snee, een operatie of een ziekte, of 2) door een verhoging van het stresshormoon adrenaline, bijvoorbeeld simpelweg als gevolg van hard lopen of fietsen.

Het stollingsproces moet natuurlijk niet spontaan in de bloedvaten optreden, dat zou namelijk tot trombose leiden. Daarom wordt het uitscheiden van de von Willebrand factor strikt gereguleerd: von Willebrand factor wordt eerst opgeslagen in de endotheel cellen in speciale blaasjes, genaamd *Weibel-Palade lichaampjes*. Pas na prikkeling van of schade aan het endotheel zullen deze Weibel-Palade lichaampjes naar het cel-oppervlak gaan, daarmee fuseren en zodoende hun inhoud in de bloedbaan uitscheiden.

Een normaal functionerend bloedstollingsproces is van vanzelfsprekend van levensbelang. Wanneer de von Willebrand factor niet goed functioneert of wanneer er te weinig von Willebrand factor aangemaakt of uitgescheiden wordt, kan het bloed niet goed stollen. Patiënten die lijden aan de zogenaamde 'ziekte van von Willebrand' kunnen vaak ernstige bloedingen krijgen en daaraan zelfs overlijden. Er zijn verschillende defecten van de von Willebrand factor aangetoond die leiden tot het slecht functioneren van dit eiwit. Wat echter zelden is beschreven, is de manier waarop Weibel-Palade lichaampjes de von Willebrand factor door de cel transporteren en in de bloedbaan uitscheiden. In dit proefschrift heb ik getracht de hele "levenscyclus" van de Weibel-Palade lichaampjes in kaart te brengen, vanaf het ontstaan in de cellen, via het transport door de cel tot het vrijlaten van hun inhoud na prikkeling van de cel.

Hoofdstuk 1 geeft achtergrond informatie over de aanmaak, ontwikkeling en uitscheiding van het tot dusver belangrijkste eiwit in de Weibel-Palade lichaampjes: de von Willebrand factor. Het belang van eiwit opslag in Weibel-Palade lichaampjes wordt hierin geintroduceerd en daarmee vormt dit hoofdstuk de basis voor de volgende hoofdstukken, waarin de verschillende aspecten van de levenscyslus van de Weibel-Palade lichaampjes verder worden bestudeerd.

De eerste stap in de levenscyclus van de Weibel-Palade lichaampjes is de selectie van de eiwitten die samen met de von Willebrand factor in de Weibel-Palade lichaampjes opgeslagen worden. Het is bekend dat de von Willebrand

factor belangrijk is om bijvoorbeeld het eiwit P-selectine mee de Weibel-Palade lichaampjes in te 'lokken'. In **hoofdstuk 2** van dit proefschrift wordt de rol van de von Willebrand factor in de selectie procedure bestudeerd van een ander belangrijk eiwit: het signalerings-eiwit interleukine-8 (IL-8). Het blijkt dat in normale endotheel cellen interleukine-8 samen met de von Willebrand factor opgeslagen in Weibel-Palade lichaampjes wordt. In bepaalde endotheel cellen die geen von Willebrand factor kunnen maken, wordt interleukine-8 wel gemaakt maar niet in Weibel-Palade lichaampjes opgeslagen. Als deze cellen door middel van gentherapie weer von Willebrand factor gaan produceren, verschijnt interleukine-8 ook weer in de Weibel-Palade lichaampjes.

Na deze eerste stap, waarin eiwitten zoals de von Willebrand factor selectief worden opgeslagen in de Weibel-Palade lichaampjes, worden de gevulde Weibel-Palade lichaampjes door de endotheel cellen getransporteerd. Door de von Willebrand factor met een groen fluorescerend eiwit te markeren was het mogelijk om het transport van de Weibel-Palade lichaampjes door de cel te volgen met behulp van een fluorescentie microscoop [**hoofdstuk 3**]. Deze aanpaak bracht aan het licht dat rustende endotheel cellen twee soorten Weibel-Palade lichaampjes bevatten. Sommige liggen bijna onbeweeglijk stil in de cel terwijl andere Weibel-Palade lichaampjes als het ware willekeurig door de endotheel cel heen bewegen en regelmatig van richting veranderen. Het lijkt erop dat de Weibel-Palade lichaampjes 'volwassen' worden tijdens dit transport en dat de gerijpte Weibel-Palade lichaampjes klaar liggen vlak onder het celoppervlak om zodra er een signaal komt, hun inhoud in de bloedbaan uit te storten. Wanneer een signaal gegeven wordt dat beschadiging van het bloedvat naabootst, fuseren alle Weibel-Palade lichaampjes met het celoppervlak en komt de inhoud vrij. Met behulp van de groene fluorescente markering is aangetoond dat het vrijkomen van de von Willebrand factor (en de rest van de inhoud van de Weibel Palade lichaampjes) inhoud erg langzaam gaat. Het gevolg hiervan is dat er op die plekken tijdelijk een hoge concentratie von Willebrand factor, IL-8 en P-selectine aanwezig is: de 'hot spots'. Dit zou belangrijk kunnen zijn voor het aantrekken van de bloedcellen die bij de stolling betrokken zijn en de schade komen herstellen. Wanneer echter een signaal gegeven wordt dat stress nabootst, dan fuseert maar een deel van de Weibel-Palade lichaampjes met cel celoppervlak. De rest beweegt weg van het celoppervlak en gaat naar het centrum van de cel. Hierdoor zou er voorkomen kunnen worden dat er bloedcellen worden opgeroepen die stolling gaan geven terwijl er geen beschadiging is.

In **hoofdstuk 4** wordt dieper ingegaan op de rol van een klein eiwitje, RalA genaamd, bij het vrijkomen van Weibel-Palade lichaampjes aan het celoppervlak. RalA zit vast aan de Weibel-Palade lichaampjes en er wordt vermoed dat RalA betrokken is bij de koppeling van de Weibel Palade lichaampjes met de cel mem-

braan tijdens de fusie. Het activeren van endotheel cellen zorgt ervoor dat RalA tijdelijk actief wordt. Deze actieve fase gaat gepaard met het vrijkomen van von Willebrand factor. Daarnaast blijken endotheel cellen die door middel van gentherapie actief RalA bevatten, continu Weibel-Palade lichaampjes uit te storten alsof ze een signaal hebben gekregen. Deze resultaten ondersteunen het vermoeden dat RalA waarschijnlijk betrokken is bij het gereguleerd vrijkomen van Weibel-Palade lichaampjes.

Zoals reeds eerder is gezegd bevatten Weibel-Palade lichaampjes een mengsel van eiwitten die een groot aantal functies in het lichaam hebben. In het tweede deel van dit proefschrift is de biologische functie van een van deze eiwitten, de von Willebrand factor propeptide, bestudeerd. **Hoofdstuk 5** is een introductie voor dit deel van het werk en illustreerd de mogelijke toepassing van dit propeptide als maat voor schade aan de endotheel cellen. Het meten van bloedspiegels van zowel de von Willebrand factor als van de propeptide maakt het mogellijk om acute en chronische beschadiging aan endotheel cellen te onderscheiden. Daarnaast blijkt de biologische rol van de propetide veel diverser dan voorheen werd aangenomen.

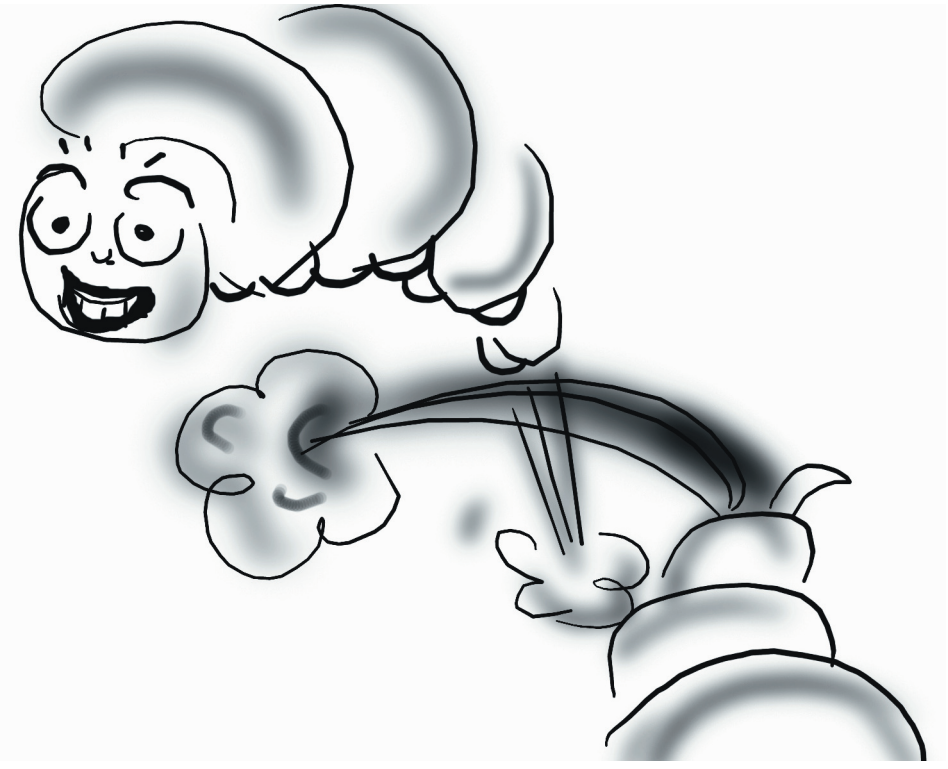
Het gebruik van de von Willebrand factor propeptide als maat voor schade aan de endotheel cellen wordt in **hoofdstuk 6** geillustreerd aan de hand van de ernstige bloedziekte thrombotic thrombocytopenic purpura (TTP), waarvan nog grotendeels onbekend is hoe de ziekte ontstaat. De bloedspiegels van dit eiwit werden gemeten in patienten bij opname in het ziekenhuis, op moment van (tijdelijk) herstel en bij het opvlammen van de ziekte. De resultaten suggereren dat endotheel activatie niet de primaire oorzaak van deze ziekte is, maar dat het eerder een consequentie is van het ziekteproces. Deze gegevens illustreren hoe het meten van bloedspiegels van eiwitten afkomstig uit Weibel-Palade lichaampjes kan helpen in het ontrafelen van onbekende ziekteprocessen.

Een andere aspect van de von Willebrand factor propeptide is de mogelijke rol die het speelt in het aantrekken van cellen tijdens ontstekingsprocessen. In **hoofdstuk 7** is beschreven dat de propeptide een bijdrage levert aan het aantrekken van bepaalde cellen uit het bloed (neutrophielen) door het signalerings-eiwit interleukine-8. Dit interleukine-8 wordt samen met de von Willebrand factor propeptide opgeslagen in Weibel-Palade lichaampjes (zie hoofdstuk 2) en ook tezamen uitgescheiden. De propeptide heeft zelf niet of nauwlijks aantrekkingskracht voor bloedcellen maar de gegevens uit dit hoofdstuk laten zien dat het de aantrekkingskracht van interleukine-8 versterkt. Op deze manier werkt de von Willebrand propetide samen met interleukine-8 om na activatie van de endotheel cellen, de juiste bloedcellen naar de beschadigde plek te loodsen.

Samengevat worden er in dit proefschrift verschillende aspecten van de levenscyclus van Weibel-Palade lichaampjes beschreven. Deze levencyclus bestaat

uit verschillende fasen met een opmerkelijke complexiteit, die nog maar ten dele ontrafelt is. De von Willebrand factor propeptide, die opgeslagen wordt in de Weibel-Palade lichaampjes, blijkt naast een rol in de endotheel cellen ook een belangrijke biologische rol te vervullen na uitscheiding in de bloedbaan. De gegevens in dit proefschrift helpen de levenscyclus van de Weibel-Palade lichaampjes beter te begrijpen en onderstrepen eens te meer de cruciale rol van Weibel-Palade lichaampjes tijdens bloedstolling, trombose en ontstekingsreacties.

DANKWOORD



MET DANK AAN...

Eindelijk!! Het boekje is af! Het kostte werkelijk een hoop bloed, zweet en tranen. Ondanks de turbulente AIO periode is toch dit prachtig boekje (al zeg ik het zelf) tot stand gekomen. Op deze plek wil ik alle mensen bedanken -al dan niet direct betrokken met dit onderzoek- zonder wie dit resultaat misschien niet mogelijk zou zijn geweest.

THANKS TO....

Finally!! The thesis is finished! This work required literally blood, sweat and tears. Despite this turbulent PhD period, this beautiful book has been produced. In this section, I would like to express my gratitude towards all the people that contributed -whether or not professionally- to the production of this thesis and without whom this result would perhaps been impossible.

Allereerst wil ik de mensen bedanken die nauw betrokken waren bij mijn onderzoek: mijn eigen onderzoeksgroepje, Jan, Herm-Jan, Martine, Erica en Ria. Onze maandag besprekingen waren altijd heel plezierig en informeel. Het bespreken van ieders onderzoeksresultaten en het uitwisselen van ideeën voor toekomstige proeven werd in een informele sfeer gedaan. Ze hoorden ons vanuit andere kamers vaak lachen. **Jan**, ondanks je moeite om knopen door te hakken, heb ik jouw hulp bij het tot stand komen van dit proefschrift heel erg gewaardeerd. Dankjewel voor de lange en zinnige discussies en voor je oprechte betrokkenheid. Je deelname aan de sociale gelegenheden zijn me goed bijgebleven. **Herm-Jan**, bedankt voor je nuchtere kijk, je goede adviezen en je bereidheid om altijd te helpen. **Ria**, jij hoorde bij ons groepje in mijn eerste jaar. Je hebt me ingewerkt in de VWF en propeptide wereld. Ondanks je overstap naar Koen's groepje is er altijd een goede band gebleven. Onze gemeenschappelijke schaatscursus vond ik heel leuk, 's avonds in het donker naar de Jaap Edenbaan rijden, daar lekker erwtensoep eten en dan een uurtje glijden op schaatsen of (in mijn geval) op m'n kont. We moeten maar snel weer eens afspreken. **Erica**, je was essentieel voor het afronden van mijn proefschrift. Ik ben je heel erg dankbaar voor je inzet en het feit dat ik altijd op je kon rekenen, niet alleen voor het praktische gedeelte maar ook voor het brainstormen en je morele steun. Ook vind ik het heel gaaf dat je mijn paranimf wilde zijn. **Gosia**, I would like to thank you for all your help during your stay in our lab. I am sure that your extraordinary drive will make you succeed in your present work.

Om een promotie-onderzoek tot een einde te kunnen brengen zijn er meer aspecten van belang dan het onderzoek alleen. Tijd is een essentieële factor voor een AIO. **Koen**, jij hield de tijdsplanning altijd goed in de gaten. De fameuze tijdschemas werden de leidraad voor elke AIO meeting. **Jan Voorberg**, jij was (en

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Thalia

CURRICULUM VITAE

Thalia Romani de Wit was born on January 7th, 1973 in Fontenay sous Bois, France. In 1991 she completed her secondary school education at the Lycée Français d'Alicante (Spain) passing both the French (Baccalaureat D) and Spanish (Selectividad Ciencias) final exams. In September of the same year, she started her masters degree in Biology at the Leiden University, graduating in August 1997. During this period she studied for one year Biology at the University of Alicante and obtained working experience at the Institue of Molecular Plant Sciences, Leiden University (supervisors Dr. L. Dekkers and Prof. Dr. B. Lugtenberg), the Wellcome Ocular Genetics Unit, Genetics Department at the Trinity College in Dublin (supervisors Dr. J. Farrar and Prof. Dr. P. Humphries) and at the Department of Immunohaematology and Bloodbank, University Medical Center Leiden (supervisors Dr. J. van Bergen and Dr. F. Koning).

From January 1998 until January 2002 she was appointed as PhD student at the department of Plasma Proteins (previously dept. of Blood Coagulation) of Sanquin Research at the CLB (Amsterdam). During this period the work described in this thesis was performed, under the supervision of Dr. J. van Mourik and Prof. Dr. K. Mertens. From March 2002 onward, she works as secretary at Hara, sector of the Municipal Health Service, Leiden.

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