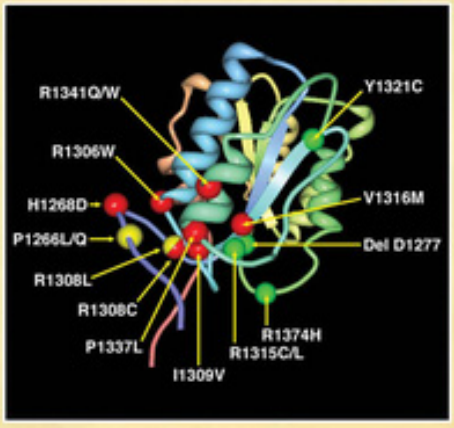
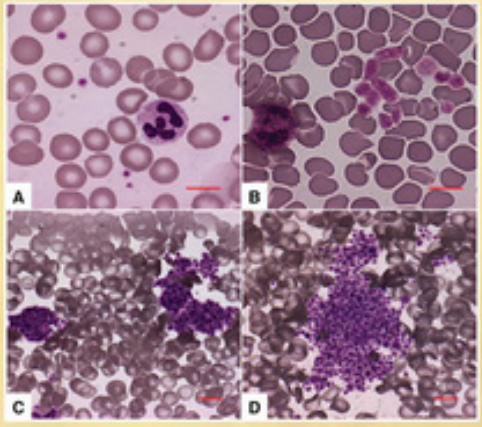
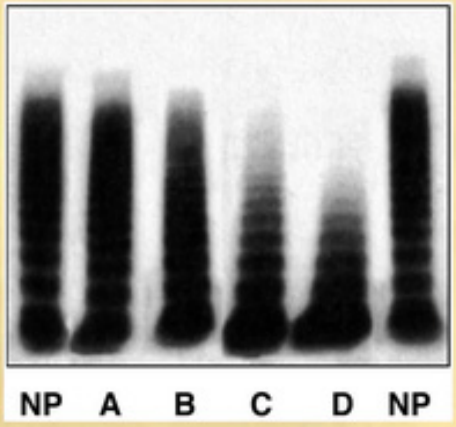
SECOND EDITION

TEXTBOOK OF VON WILLEBRAND DISEASE

BASIC AND CLINICAL ASPECTS







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Textbook of von Willebrand Disease

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Basic and Clinical Aspects

Second Edition

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This edition first published 2024

Edition History
Blackwell Publishing Ltd (1e, 2011)

Registered Offices
John Wiley & Sons, Inc., 111 River Street, Hoboken, NJ 07030, USA
John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK

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Library of Congress Cataloging-in-Publication Data applied for
ISBN 9781119419488 (Hardback)

Cover Design: Dr. Luigi Ghilardini
Cover Images: Courtesy of Augusto B. Federici and Luigi Ghilardini

Set in 9/11.5pt SabonLTStd by Straive, Pondicherry, India

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Foreword

I feel very honored to have been asked to write the foreword to this textbook on von Willebrand disease (VWD). I am now the oldest living scientist to have experience in this area, and thus it may be of interest for readers to learn about some early experiences that I shared with the late Dr. Inga-Marie Nilsson, which I have described below. Since I started working with VWD in the mid-1950s, there has been enormous progress in the management of the disease in terms of knowledge about mechanisms, treatment, and underlying genetics. We have been able to follow this development in Stockholm because the hemophilia center here is currently responsible for the treatment of 40 patients with type 3 VWD (i.e., the most severe form).

In the 1950s, there were only a few known cases of the disease—which was mostly called "pseudohemophilia"—in addition to those cases known in the Åland Islands, where the disease was first identified by Erik von Willebrand. This was probably because most patients with type 3 VWD died young, either *in utero* or, if the patient was female and survived until puberty, as a result of menstrual bleeding. I remember some touching letters written at the end of the 19th century from a businessman to his wife, who was mostly bedridden owing to menstrual bleedings. This woman was an ancestor of a young woman from Stockholm with type 3 VWD, who is currently living a normal family life thanks to therapy in early childhood with the Swedish fraction I-0 (which contained von Willebrand factor [VWF], factor VIII [FVIII], and fibrinogen) and later with commercial VWF-containing concentrates.

When taking a bleeding history for a female in the 1950s, it was useful to ask whether she had been scolded in school for dropping blood onto her handiwork after pricking her finger with a sewing needle. We learned that it was useful to analyze blood groups in family investigations, as we found that a healthy child who showed no sign of having inherited the disease did not share the same father as the sick sibling. We made several mistakes—one girl was transfused with platelets during severe menstrual bleeding without success, but when treated with fraction I-0, the bleeding stopped. It is possible that the platelet treatment was the reason why she later developed antibodies to VWD. At that time, there were no oral contraceptives, which have revolutionized the management of menorrhagia in patients with VWD. In this particular patient, we used testosterone and later hysterectomy (under prophylaxis of fraction I-0) to deal with the menstrual bleeding.

Persuading doctors that a patient had to be treated with a concentrate was a difficult task. I remember the case of a 13-year-old boy who developed severe head trauma as a result of falling from a bicycle. Despite the fact that the boy had a bleeding chart saying that he should be treated immediately in the event of a trauma and the fact that I informed the doctor that the usual signs do not develop in bleeders immediately but sometimes several days later, the doctor refused to treat the boy with concentrates and he died from severe brain hemorrhage.

In 1958, we started prophylactic treatment for patients with hemophilia to avoid joint destruction. However, it was not until many years later that we realized that patients with type 3 VWD also required prophylaxis; therefore, some of them developed joint disabilities. We also did not know that the concentrates with which we treated our patients could contain hepatitis C virus, which has led to the premature death of some patients.

This book has become a very comprehensive and useful work into which many of the authors have put great efforts to make their chapters not only informative but also easy to understand. Progress, difficulties, and alternative ways to diagnose phenotypes and genotypes are described. Molecular diagnosis of type 1, type 2 and its variants, and type 3 VWD is presented. In addition, a chapter on gene therapy looking into the future is stimulating to read. Furthermore, many authors have endeavored to include all relevant literature, which is very useful for students.

A problem with regard to historical aspects is that the nomenclature has changed from FVIII-related antigen to VWF antigen. Therefore, some of the early findings with regard to the level of VWF in patients with blood group O or A have not been observed. Nevertheless, the topic of how to proceed in diagnosis when the patient has blood group O or A has been thoroughly discussed. I have the impression that there still are problems with regard to the diagnosis of the phenotypes, particularly with regard to the diagnosis of type 1 VWD, even if pre-analytic problems are taken into account, for example, the quality of methodology and the importance of telling the patient to rest and not to run or be stressed, before blood sampling. I made a serious mistake once when analyzing changes in VWF during the menstrual cycle—the volunteers were not well informed about resting before sampling and we therefore misinterpreted the results; there are not such great variations in FVIII and VWF during the menstrual cycle as initially suggested.

When investigating families with type 3 VWD, we found that the parents and siblings who were genetic carriers of VWD only had a phenotypically mild bleeding disorder, and often, but not always, the common analyses of VWD indicated a mild disorder.

However, we recorded the usefulness of an increased ratio of FVIII/VWF:Ag for the diagnosis of what we called type 1 VWD in these families.

It must have been an enormous task for the editors to encourage all the authors to write, although possibly some welcomed the opportunity to put together their experience in a comprehensive chapter. The efforts to try to collate experience in multicenter studies on prophylaxis and diagnostic scores are very valuable and, of course, need to be supported in order to solve the many difficulties that remain in the diagnosis and management of VWD.

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Preface

Erik von Willebrand described a novel bleeding disorder in 1926, and in his original publication, he provided an impressive description of the clinical and genetic features of von Willebrand disease (VWD). In contrast to hemophilia, the epitome of inherited bleeding disorders, both sexes were affected, and mucosal bleeding was the predominant symptom. The history of VWD is fascinating because it demonstrates how good clinical observations, genetic studies, and biochemical skills can improve the basic understanding of a disease and its management. The continuous efforts of scientists and clinicians over the last 85 years have significantly furthered the understanding of the structure and function of von Willebrand factor (VWF), the protein that is absent, reduced, or dysfunctional in patients with VWD. Such basic information about VWF will undoubtedly improve both the diagnosis and the treatment of VWD. Determination of both the phenotype and the genotype is now readily available in many countries, and treatment is becoming more specific and directed by the type and subtype of VWD. Therapeutic agents must correct the dual defect of hemostasis, i.e. the abnormal platelet adhesion due to reduced and/or dysfunctional VWF and the associated low level of factor VIII (FVIII). Desmopressin (DDAVP) is the treatment of choice for type 1 VWD because it induces the release of VWF from cellular compartments. VWF concentrates that are virally inactivated, with or without FVIII, are effective and safe in patients unresponsive to DDAVP. The recombinant VWF was under evaluation in clinical trials when the first edition of this book was published and is now used to manage VWD patients in most developed countries. Retrospective and prospective clinical studies, including bleeding history and laboratory markers for diagnosis, as well as the use of DDAVP and VWF concentrates to treat or prevent bleeding in patients with VWD, have been essential to provide international guidelines for the management of VWD such as those published in 2021 by ASH/ISTH/WFH societies. More efforts should be devoted to promote awareness of VWD especially in developing and low-income countries where high costs for laboratory diagnosis cannot be afforded. More simplified clinical and diagnostic approaches should be designed.

In 2026, we are going to celebrate the first century since the original description of VWD by Erik von Willebrand. The second edition of this book has been organized to report the most updated basic and clinical aspects of inherited and acquired defects of VWF. All the important advances have been described in more chapters by the most experts who have contributed directly to the improved management of VWD patients. For these reasons, the editors decided to propose a new title for this second edition of the book: *Textbook of von Willebrand Disease: Basic and Clinical Aspects*. The editors hope that a book specifically devoted to VWD can be useful to the hematologists of the 21st century who would like to manage VWD patients in a more comprehensive way using the most updated and evidence-based recommendations.

The editors would like to dedicate this *Textbook on VWD* to three pioneers in VWD research who made pivotal and original contributions to this field: Arthur Bloom, Inga Maria Nilsson, and Theodore S. Zimmerman. Another special dedication must be attributed to J. Evan Sadler who pioneered the study of the two proteins VWF/ADAMTS13 involved in VWD basic mechanisms and classification. Their lifelong devotion to research on VWD and other bleeding disorders should stimulate further studies on these topics of hematology.

The Editors
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16 January 2024

1 Historical perspective on von Willebrand disease

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Introduction

The history of von Willebrand disease (VWD) and its causative factor, the von Willebrand factor (VWF), spans almost a century and was recently comprehensively reviewed by the late professor Birger Blombäck, who described the first publication by Erik von Willebrand [1], the gene cloning in 1985, and the discovery of the specific metalloprotease, ADAMTS13 [2], which degrades VWF. The purpose of this review is to describe the early history of the understanding of the disease and the first steps in the replacement therapy for its severe forms. Also, we describe in greater detail the findings of a group of different families investigated on the Åland Islands.

The scientist of the disease

Erik Adolf von Willebrand (Figure 1.1) was born in Vasa, Finland, in 1870. He qualified as a medical doctor in 1896 and specialized initially in physical therapy and later in internal medicine in Helsinki. Erik von Willebrand devoted much of his professional life to an interest in blood, especially its coagulation properties. In 1899, he defended a doctoral thesis that dealt with his investigation of the changes that occur in blood following a serious hemorrhage. From 1908 until his retirement in 1935, Erik von Willebrand worked at the Deaconess Institute in Helsinki, where he headed the Department of Internal Medicine between 1922 and 1931. Erik von Willebrand was known for his modesty and integrity, and in his obituary it was said that he "usually preferred to discuss his observations of nature rather than his personal achievements." He died in September 1949, at the age of 89 years.

First description of the disease: the Åland family

In 1926, Erik von Willebrand first described the inherited bleeding disorder in *Finska Läkaresällskapets Handlingar* (in Swedish). He identified features that suggested that this disease was distinct from classic hemophilia and other bleeding disorders known at the time, such as anaphylactoid purpura, thrombocytopenic purpura, and hereditary thrombasthenia, described by Glanzmann. What differentiated this bleeding disorder from classic hemophilia was that it was not frequently associated with muscle and joint bleeding, and it affected both women and men. He stressed that a prolonged bleeding time was its most prominent characteristic. He concluded that the condition was a previously unknown form of hemophilia, and called it "hereditary pseudohemophilia." Erik von Willebrand also discussed the pathogenesis of the condition and felt that the bleeding could best be explained by the combined effect of a functional disorder of the platelets and a systemic lesion of the vessel walls.

The original observations leading to this new disease were made in several members of a large family (identified as family S) living on the island of Föglö in the Åland archipelago in the Baltic Sea. The index case was a girl aged 5 years, named Hjördis S, who had marked and recurrent bleeding tendencies and was brought to Helsinki for consultation. Both her mother and father were from families with histories of bleeding. The girl was the ninth of 11 children, of whom 7 had experienced bleeding symptoms. Four of her sisters had died from uncontrolled bleeding at an early age. Hjördis herself had experienced several severe episodes of bleeding from the nose and lips and following tooth extractions, as well as bleeding in her ankle. At the age of 3 years, she bled for 3 days from a deep wound in her

Textbook of von Willebrand Disease: Basic and Clinical Aspects, Second Edition. Edited by Augusto B. Federici, Erik E. Berntorp, David Lillicrap, and Robert R. Montgomery.

upper lip. The bleeding was so severe that she almost lost consciousness and had to be hospitalized for 10 weeks. At the age of 14 years, Hjördis bled to death during her fourth menstrual period.

Hjördis came from a large family (Figure 1.2). Intrigued by their history, Erik von Willebrand studied the family further with the help of coworkers. He published the pedigree and his clinical and laboratory evaluation in his 1926 paper. He found that 23 of the 66 family members had bleeding problems. The most prominent problem among the affected family members was mucosal bleeding: epistaxis, followed by profuse bleeding from oral lesions, easy bruising, and, in females, excessive bleeding during menstruation and childbirth. Intestinal bleeding had been the cause of death at early ages in some family members.

Figure 1.1 Erik von Willebrand.

In further studies, Erik von Willebrand found two families related to Hjördis S and one unrelated family in whom bleeding symptoms similar to those observed in Hjördis were common [3, 4]. In the 1930s, Jürgens, together with von Willebrand [5, 6], reinvestigated the patients in Åland and concluded that the disease was due to some impairment of platelet function, including platelet factor 3 deficiency. This observation led to the disease being called von Willebrand–Jürgens thrombopathy, and, although this condition is not officially recognized today, von Willebrand did not dismiss the notion that factors in blood plasma might also be important in the pathogenesis of the disease.

Other early clinical reports

In 1928, Dr. George R. Minot of Boston described five patients from two families with prolonged bleeding times and symptoms similar to the Åland family members. This may have been one of the first descriptions of VWD [7–9]. In the following years, numerous cases similar to those described by von Willebrand were reported, usually under the name of pseudohemophilia. In 1953, Alexander and Goldstein [10] found a dual defect in two patients with hereditary pseudohemophilia. They confirmed the earlier findings of prolonged bleeding time, normal platelet count and function, and abnormal nail bed capillaries. However, they also found a decreased FVIII level (5–10% of normal) and they observed a prolonged coagulation time that was normalized by normal plasma. The prolonged bleeding time, however, was not normalized and this was later explained by the fact that infusion of a restricted volume of plasma does not provide a sufficient amount of VWF [11]. Larrieu and Soulier [12] also found low FVIII activity and a prolonged bleeding time in pseudohemophilia, but otherwise normal clotting factors and platelet parameters. They proposed the name von Willebrand syndrome for the condition.

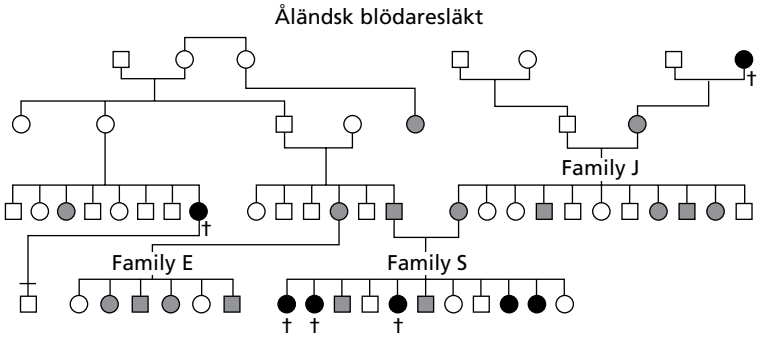

Figure 1.2 The Åland pedigree as originally described in 1926 [1]. The index case, Hjördis, is the ninth sibling in family S (Fam S). □ unaffected male; ○ unaffected female; ◐ male with mild bleeding disease; ◼ female with mild bleeding disease; ● female with severe bleeding disease; † bled to death.

The search for a new factor—the bleeding time factor

The first demonstration of the VWF was during the 1950s through a joint effort by Margareta and Birger Blombäck, working in Stockholm with the purification of fibrinogen, and Inga Marie Nilsson, who had established a clinical coagulation unit in Malmö. It was found that fibrinogen purified from Cohn fraction I of human plasma, when specifically obtained in fraction I-0 (AHF-Kabi), was heavily contaminated with an antihemophilic factor, which is plasma factor VIII (FVIII) [13].

At that time, Dr. Nilsson had a 15-year-old female patient named Birgitta who had a severe hemorrhagic diathesis. When she began to menstruate, the condition worsened and she received frequent blood transfusions. However, Birgitta developed serious side-effects from the transfusions and they were stopped. As a consequence, other treatment options had to be considered, and a hysterectomy was planned. Her coagulation evaluation had shown a prolonged bleeding time and a somewhat prolonged coagulation time but normal platelet count and function. FVIII activity was low. Since fraction I-0 had a high concentration of FVIII, it was decided that its effects should be tested in Birgitta. To the surprise of the treating physicians, not only did FVIII activity increase as expected but the bleeding time was also normalized [14]. Subsequently, a hysterectomy was successfully performed under the cover of fraction I-0. According to modern classification, this patient had type 3 VWD. She is now well and has been on regular prophylaxis with VWF concentrate for many years.

In June 1957, Inga Marie Nilsson, Erik Jorpes, Margareta Blombäck, and Stig-Arne Johansson visited Åland and studied 16 patients who had been examined 25–30 years previously by von Willebrand. No patients who had severe forms of the disease were still living. In their investigation, they found FVIII activity to be reduced in 15 of 16 cases [15]. The father of Hjördis had a normal level. The Duke bleeding time varied, with two patients having a definite prolongation and three patients having a moderate prolongation. Platelet counts were normal and, in contrast to Jürgens' earlier observation, the platelets themselves were normal with respect to platelet factor 3. One of the patients was given fraction I-0, which normalized the FVIII level and the bleeding time. It could be concluded that the Åland family had the same disease described by several other authors in Europe and the USA [16]. At the same time, Jürgens visited the islands (Erik Jorpes had told him of his team's research plan) and took samples from many of the same patients, and confirmed the decreased FVIII levels [17].

The findings by the Swedish group confirmed what had been documented in a number of Swedish families [18]. The observation was also made that FVIII increased during the first 24 hours after infusion of fraction I-0 in patients with VWD, in contrast to what is seen in hemophilia [19]. The results of fraction I-0 infusion in a patient with severe VWD are shown in Figure 1.3. The bleeding time is reduced or normalized; factor VIII clotting activity (VIII:C) increases steadily during the first 24 hours, whereas the VWF (VIIIR:Ag and VIIIR:RC according to old nomenclature) displays a pharmacokinetic profile as expected and as later shown. Control experiments and further studies [11, 20, 21] revealed that the bleeding time factor was a plasma factor not earlier described. Fraction I-0 prepared from patients with severe hemophilia A not only corrected the bleeding time in VWD, but also stimulated the production of FVIII activity, whereas fraction I-0 prepared from patients with VWD had no such effect. Purified fibrinogen had no effect on the bleeding time. Still, there was the possibility that the shortening of the bleeding time was due to platelets or platelet factors contaminating fraction I-0. This turned out to be unlikely, since the effect on bleeding time was the same whether the fraction had been prepared from platelet-rich or platelet-poor plasma. Infusion of a platelet suspension from a normal donor to a patient with VWD had no effect on either bleeding time or bleeding tendency or did injection of fraction I-0 into a patient with thrombocytopenia. From these findings, it was concluded that the impaired hemostasis in VWD was due to lack of a plasma factor, the bleeding time correcting factor, or the VWF, which occurs not only in normal plasma but also in hemophilia A plasma. This factor not only corrected the prolonged bleeding time but apparently increased the level of FVIII. Thus, platelets or platelet factors were not identical with the bleeding time factor, which had been proposed by both Rudolf Jürgens and Erik von Willebrand to be responsible, together with a vascular defect, for the bleeding diathesis. These findings have since been widely confirmed. The claim that a previously

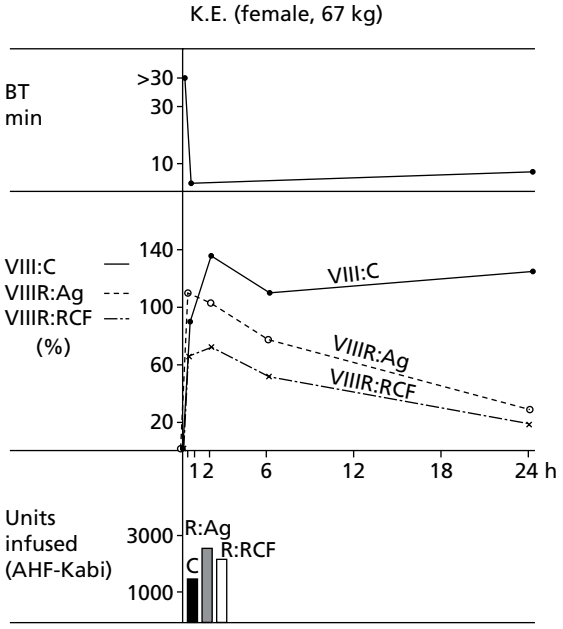

Figure 1.3 VIII:C, VWF:Ag (VIIIR:Ag), VWF:RCo (VIIIR:RCF), and Duke bleeding time (BT) in a patient with severe von Willebrand disease after infusion of human fraction I-0 (AHF-Kabi) [16]. Bleeding time is shortened and VIII:C is successively increased after the initial post-infusion peak during the first 24 hours, whereas the von Willebrand factor (VIIIR:Ag and VIIIR:RC) displays a pharmacokinetic profile as expected. Reproduced from Nilsson and Holmberg [16].

unknown factor in plasma had been discovered was communicated at the Congress of the International Society of Hematology in Rome in 1958 (see also Ref. [20]).

At first, it was not understood how a plasma factor could affect primary hemostasis and shorten the bleeding time. However, Borchgrevink [22] found decreased platelet adhesiveness *in vivo*, and Salzman [23] demonstrated decreased platelet adhesiveness to glass in VWD. Borchgrevink employed the method suggested by Hellem [24], which used a slow flow and could not discriminate between samples from patients with or without VWD. Salzman modified this method and introduced a higher flow, making it more specific for VWD. It was also shown that normal or hemophilic plasma can normalize the reduced platelet adhesiveness as well as the bleeding time in VWD [23, 25, 26]. In studies using electron microscopy, Jörgensen and Borchgrevink [27] demonstrated a decreased adhesion of platelets to disrupted endothelium in VWD. This observation indicated that the plasma factor lacking in VWD exerted its action in primary hemostasis via the platelets by enhancing their adhesiveness.

During the 1960s, cases of VWD were reported in several countries. The disease was thought to be uniform and was defined as an autosomal dominant inheritable hemorrhagic disease characterized by a prolonged bleeding time, decreased FVIII clotting activity, decreased platelet adhesiveness as measured by the Salzman method, and progressive increase of FVIII activity after infusion of plasma and FVIII concentrate [16].

However, returning to the earlier papers by Erik von Willebrand and Rudolf Jürgens, the findings on the Åland islands showed what appeared to be a discrepancy between the original family S and some of the others investigated; the original von Willebrand family having "pure" VWD while in other families there were also platelet function defects. Thus, in 1977, Dag Nyman (originally from Åland) and collaborators [28] traveled from Stockholm to Åland to undertake a thorough investigation using new laboratory methods [28]. They found that the families described as having VWD could be divided into four categories: (i) the survivors with a mild disorder from the original family S had the characteristics of type 1 VWD, that is they had similarly decreased levels of VWF:Ag and ristocetin cofactor activity in addition to normal or decreased levels of FVIII, and the platelet aggregation was normal; (ii) one family had a platelet function defect (pure cyclooxygenase defect); (iii) one family had a mixture of VWD and a cyclooxygenase defect; and (iv) one family had a platelet function defect of the aspirin type. These findings, of course, made it easier to investigate the genetic defects of the original VWD (family S).

In the beginning of the 1990s, Zhang and collaborators [29] investigated the DNA sequence of 24 patients with type 3 VWD living in Sweden. They found a cytosine deletion in exon 18 of the *VWF* gene in most of those of Swedish origin and an insertion in exon 28 in those of Finnish origin. Most patients with type 3 VWD were homozygous or double heterozygous for the mutations. Most of the parents had type 1 VWD and were heterozygous. As the Åland population is primarily of Swedish origin, the researchers also investigated family S and found that the surviving members who had type 1 VWD were heterozygous with respect to the mutation in exon 18. There was a small boy with severe VWD whose family was related long ago to another family with VWD from Åland. He was homozygous for the mutation in exon 18 [30].

The end of the beginning

After the publication by Erik von Willebrand in 1926, it took some 30 years until it was clear that a new plasma factor responsible for the hemostatic impairment in VWD had been detected. At that time, a factor concentrate had been produced that was effective in the replacement of VWF: fraction I-0 (or, later, AHF-Kabi). Studies using this concentrate and concentrates purified from different types of bleeding disorders, helped scientists to find and prove the presence of the VWF. This was the end of the beginning.

In 1971, VWF was first detected immunologically and named "FVIII-related antigen" [31]. Since 1985, the VWF has been cloned [32–35], the primary amino-acid sequence has been determined [36], and the complex molecular structure and multiple functions are becoming understood in detail. The metalloprotease ADAMTS13 that cleaves VWF was discovered in 2001 [2]. VWD is no longer a uniform disease [37]. The treatment armamentarium has been developed and includes prophylactic treatment with concentrates, especially in type 3 VWD. It includes desmopressin for most milder cases, new concentrates [38, 39], including recombinant VWF [40], for therapeutic use.

Recent scientific visits to Åland Islands

Many scientific visits have been organized since the report by Erik von Willebrand and pioneering visits were performed by Margareta Blombäck, Inga Marie Nilsson, and others during the 1950s when blood sampling was performed from the family members concerned. In more recent years, scientific conferences have been organized, among them in 2016—90 years after the first case of VWD was diagnosed in a patient from the Åland Islands in 1926 [41]. Sixteen experts in the field from Europe and North America convened to share knowledge and expertise on current trends and challenges in VWD. Topics included the storage and release of VWF, epidemiology and diagnostics in VWD, treatment of VWD, angiogenesis, and VWF inhibitors. Delegates also visited the house where Hjördis was living until her far too early death and her grave. A more obvious and realistic historic journey of a previously deadly bleeding disorder cannot be described.

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2 Biosynthesis and organization of von Willebrand factor

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Introduction

Von Willebrand factor (VWF) is a large multimeric adhesive plasma glycoprotein that is synthesized in megakaryocytes and endothelial cells [1]. Decreased levels or defective function of VWF cause the most common inherited bleeding disorder, von Willebrand disease (VWD), which has a prevalence estimated by some to be as high as 1% [2]. The primary function of VWF is to promote platelet binding to subendothelial tissue at the site of a vascular injury. VWF also mediates platelet–platelet interactions, promoting further clotting. A second critical role for VWF is that it serves as the carrier protein for coagulation factor VIII (FVIII), protecting it from proteolytic degradation in plasma. The biosynthesis and organization of VWF involve a complex intracellular pathway; defects at any point in this pathway may contribute to the decreased plasma VWF level or dysfunction that causes VWD.

Terminology

It was not known until the 1970s that VWF and FVIII are different proteins with distinct functions [3]. VWF is the carrier protein for FVIII in plasma; thus, these two proteins are intimately associated with one another and may copurify when isolated from plasma, leading to erroneous identification. Earlier reports referred to VWF as "FVIII-related antigen."

A second antigen is absent from plasma and platelets in patients with severe VWD. This additional antigen was historically called von Willebrand antigen II (VW AgII) but is now termed the VWF propeptide (VWFpp) [4, 5]. VWFpp was subsequently found to be synthesized in endothelial cells together with VWF [6]. When the *VWF* gene was later cloned, it was discovered that the N-terminal sequence of pro-VWF was identical to that of VWFpp [7]. It is now well-established that VWFpp is the 741-amino acid propeptide of VWF that is cleaved from VWF, stored with it in Weibel–Palade bodies of endothelial cells and α-granules in megakaryocytes, and released with VWF into plasma from these storage organelles.

Molecular biology of VWF

The *VWF* gene

The coding sequence for VWF was first identified in 1985 by four independent groups [8–11]. The VWF mRNA was shown to be approximately 9 kb in size. After the coding sequence for VWF was identified, the entire *VWF* gene was cloned [12]. The gene has been localized to chromosome 12. The complete intron/exon sequence has been determined, and the 52 exons span approximately 178 kb. The size of exons varies between 40 bp and 1.4 kb for exon 28. A second, partial VWF sequence has been identified on chromosome 22. This pseudogene shows 97% homology with the authentic *VWF* gene on chromosome 12. However, the occurrence of several stop codons within the coding sequence indicates that this gene is not expressed in humans. The presence of this second pseudogene can cause problems in identifying sequence abnormalities in patients with VWD, although this can be overcome with the proper design of sequencing primers.

VWF domain structure

The open reading frame of the VWF cDNA predicts a 2813-amino acid protein as the primary translation product (Figure 2.1a). The transcriptional start site is located 245 nucleotides upstream of the initiator methionine. Using the current numbering system, the initiating methionine codon is defined as nucleotide number one, and the corresponding methionine residue is defined as amino acid 1. The N-terminal segment of

Textbook of von Willebrand Disease: Basic and Clinical Aspects, Second Edition. Edited by Augusto B. Federici, Erik E. Berntorp, David Lillicrap, and Robert R. Montgomery.

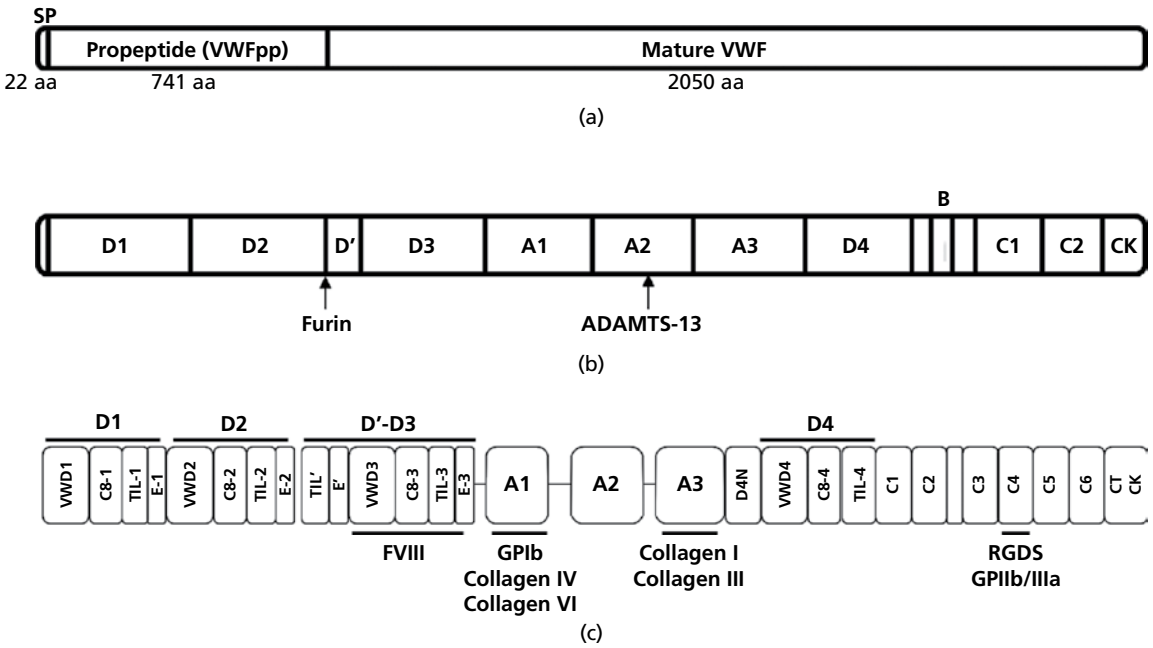

Figure 2.1 Domain structure of von Willebrand factor (VWF). (a) VWF is synthesized as pre–pro-VWF containing a 22-amino acid (aa) signal peptide (SP), 741-amino acid propeptide (VWFpp), and 2050-amino acid mature VWF molecule. (b) The original domain structure of VWF protein consists of a series of repeated homologous A, B, C, and D domains with VWFpp consisting of D1 and D2 domains. Mature VWF comprises D′–D3–A1–A2–A3–D4–B1–B2–B3–C1–C2–CK domains. VWFpp is proteolytically cleaved from mature VWF at amino acid 763 by the enzyme furin. In plasma, VWF is proteolytically cleaved by the ADAMTS13, and the cleavage site is located in the A2 domain, between amino acids 1605 and 1606. (c) VWF domain structure was recently updated and re-annotated using electron microscopy structural data. VWF contains sites for interaction with coagulation factor VIII, platelet glycoprotein GPIb, types I, III, IV, and IVcollagen, and platelet glycoprotein GPIIb/IIIa.

VWF includes a 22-amino acid signal peptide and a 741-amino acid propeptide (VWFpp), followed by a 2050-amino acid mature VWF molecule (Figure 2.1a). The propeptide is proteolytically removed from the mature VWF protein in the Golgi, presumably by the enzyme furin (PACE). Historically, the VWF protein was reported to be composed of a series of repeated homologous domains that are termed A, B, C, and D domains (Figure 2.1b). The propeptide contains two D domains, D1 and D2. The mature VWF protein is composed of D′–D3–A1–A2–A3–D4–B1–B2–B3–C1–C2–CK domains. More recently, the VWF domain structure was updated to assign specific modules, and these modules were related to structure using electron microscopy (Figure 2.1c). The updated D domains are assemblies of small modules. The B and C domains are now re-annotated as 6 tandem von Willebrand C (VWC) and VWC-like domains [13].

Several functions of VWF have been mapped to specific VWF domains (Figure 2.1c). The D′–D3 domains are important in binding FVIII to VWF [14, 15]. The VWF A1 domain is essential in binding VWF to platelets through the platelet receptor glycoprotein, GPIbα, and also contains binding sites for heparin and types IV and VI collagen [16–20]. The A2 domain contains the cleavage site for post-secretion processing of VWF by the VWF-cleaving protease, ADAMTS13 [21–23]. The A3 domain has been reported to contain a binding site for collagen types I and III [24–26]. The C1 domain contains an Arg-Gly-Asp-Ser (RGDS) sequence that may bind the platelet glycoprotein GPIIb/IIIa [27].

Mutations in these binding domains have been found in patients with VWD. Patients with type 2N VWD have decreased plasma VWF levels as a result of substantially impaired FVIII binding to VWF [1, 2]. Mutations have been identified in the D′–D3 domains in several of these individuals [28–34]. Type 2B VWD is characterized by a loss of plasma high-molecular-weight multimers resulting from increased binding of VWF to platelets. Mutations in type 2B VWD patients have been identified in the A1 domain, which contains a binding site for the platelet glycoprotein GPIb [35–39]. Other mutations in the A1 domain prevent the binding of VWF to platelets, characteristic of type 2M VWD [40–42]. Individuals with type 2A VWD have decreased high-molecular-weight multimers and a platelet-binding function. Type 2A VWD results from at least two distinct mechanisms: defective multimerization and secretion, or increased susceptibility to proteolysis by ADAMTS13 [43, 44]. Mutations causing increased cleavage by ADAMTS13 are likely to be identified in the A2 domain, which contains the cleavage site for ADAMTS13 proteolysis [45–50]. Mutations causing defective multimerization and secretion have been identified in the D1, D2, D′–D3, A1, A2, and CK domains of VWF [51–57]. Mutations associated with impaired binding of VWF to types I, III, IV, and VI collagen have also been identified and fall under the type 2M VWD subtype [20, 58–62].

VWF promoter

The VWF promoter is complex, and several upstream regulatory elements controlling VWF expression have been identified. A number of consensus sequences for *cis*-acting elements have been defined in the upstream promoter region and in the first exon, including two GATA-binding sequences [63]. The endothelial cell-specific regulation of VWF expression has been investigated and found to be controlled by a repressor—derepressor mechanism involving an NF1 binding site, an Oct-1 binding site, and Ets transcription factors [63–69]. In addition to endothelial cell-specific expression, there are also complex pathways of transcriptional regulation through vascular bed-specific regulation. This vascular bed-specific regulation of the endothelial cell *VWF* gene is controlled by the tissue microenvironment [70]. The E4BP4 transcriptional repressor has also played a role in the cell type-specific regulation of VWF expression [71]. Additionally, single nucleotide polymorphisms (SNPs) in the VWF regulatory region have been identified that are associated with plasma VWF Ag levels, including the nucleotides −1793, −1234, −1185, and −1051. The regulation of VWF expression is complex and controlled by cell-specific and vascular bed-specific regulatory elements.

Cell biology of VWF

VWF is synthesized exclusively in endothelial cells and megakaryocytes [72, 73]. The processing of VWF involves a very complicated sequence of events. Most of the studies on VWF processing, assembly, and secretion have utilized cultured endothelial cells or transfected mammalian cells, although some studies on VWF expression in megakaryocytes have also been reported [5, 50, 72–87]. The processing of VWF in endothelial cells and megakaryocytes appears to be similar, as VWF from both sources is structurally alike; however, differences in glycan composition have been identified [88]. In both endothelial cells and platelets produced by megakaryocytes, VWF forms high-molecular-weight multimers and is packaged in secretory vesicles; Weibel–Palade bodies in endothelial cells; and α-granules in megakaryocytes [74, 89]. Much of our understanding of VWF processing comes from expression studies using a variety of mammalian cells, as summarized in the following paragraphs.

VWF processing and dimerization in the endoplasmic reticulum

When moving through the cell's secretory pathway, VWF undergoes extensive intracellular modifications (Figure 2.2). VWF is initially synthesized as pre–pro-VWF containing a signal peptide, propeptide, and mature VWF polypeptide. In the endoplasmic reticulum (ER), the signal peptide is removed, the pro-VWF protein is folded, and disulfide bonds are formed (Figure 2.2). VWF is a cysteine-rich protein with 64 cysteine residues in VWFpp and 170 cysteines in the mature VWF protein [7]. In the secreted VWF protein, all cysteines appear to be involved in disulfide bonds, as historically, no free sulfhydryls have been detected. However, some studies employing more sensitive techniques suggest that there may indeed be some reactive unpaired cysteines in plasma VWF [83, 90]. While the mapping of disulfide bonds has been accomplished for some cysteines in VWF, the majority of disulfide mapping is unresolved [13, 91–95]. Given the number of cysteines in the full-length VWF, the process of protein folding and disulfide bonding must be exceptionally complicated.

In the ER, the pro-VWF subunits form carboxyl-terminal dimers (Figures 2.2 and 2.3). This dimerization involves the last 151 amino acids of the mature VWF protein [86, 95]. Voorberg *et al*. have demonstrated that recombinant VWF that lacks these 151 amino acids fails to dimerize and is proteolytically degraded in the ER [86]. Thus, these carboxyl-terminal sequences may serve a role in retaining monomers in the ER until they are either dimerized or degraded. The last 90 residues of VWF comprise the CK domain, which contains a sequence homologous to the cysteine knot family of proteins. The common characteristic of this family of proteins is their tendency to dimerize through the formation of disulfide bonds. Further evidence of the importance of this region to VWF dimerization has been provided by an investigation of patients with VWD who have VWF structural abnormalities. Several mutations have been identified in this region of VWF, including C2362F, C2739Y, C2754W, C2773R, and A2801D variants. Expression studies using recombinant VWF variants demonstrated a defective formation of VWF dimers, indicating the critical role of the carboxyl-terminal region in dimerization [96–99]. While the importance of the C-terminal portion of VWF in dimerization is clear, the N-terminal has been found to be less important. The large propeptide VWFpp (pro-VWF) is not necessary for dimerization. Expression of a propeptide-deleted mature VWF (signal peptide sequence followed by mature VWF sequence) results in the secretion of a dimeric VWF protein, indicating that VWFpp is not necessary for the formation of dimers or for exit from the ER [79, 100–102].

In addition to protein folding, disulfide formation, and dimerization, the large pro-VWF is also extensively modified in the ER by the addition of N-linked glycans. The mature VWF protein contains 13 N-linked glycosylation sites, and VWFpp contains four N-linked sites [103, 104]. The O- and N-linked carbohydrates account for approximately 18–19% of the total VWF protein mass. Interestingly, the N-linked glycans of the VWF protein contain over 100 distinct glycan compositions, including ABO blood group oligosaccharides [103, 105]. Wagner and colleagues found that when human endothelial cells were metabolically labeled, it took approximately 120 minutes for VWF with complex-type oligosaccharide chains to be detected [74]. As soon as metabolically labeled VWF was detected in the cell, it was also found to be constitutively secreted. The exit of pro-VWF from the ER appears to be the rate-limiting step in VWF biosynthesis, as it is for other proteins. The exit of VWF from the ER is dependent on both glycosylation and dimerization. When N-linked glycosylation is blocked by the addition of tunicamycin to the culture medium of endothelial cells, as reported by

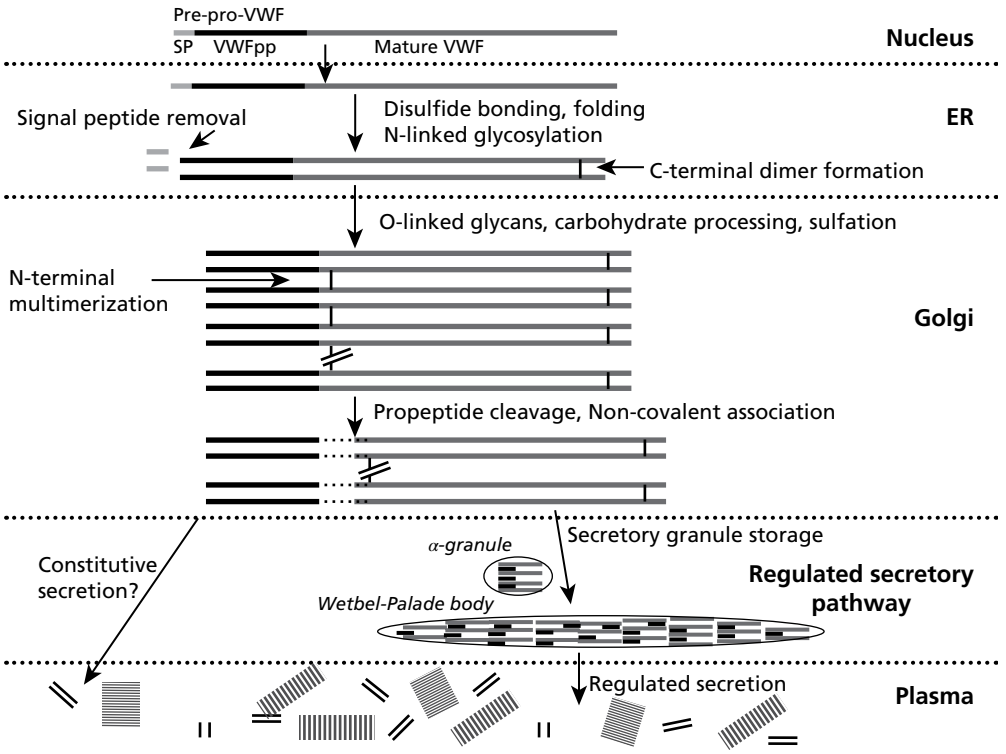

Figure 2.2 Intracellular processing of von Willebrand factor (VWF). The pathway of VWF biosynthesis and organization is depicted in this illustration. The VWF promoter is synthesized as pre–pro-VWF. In the endoplasmic reticulum (ER), VWF is folded, disulfide bonds are formed, glycosylation occurs, the signal peptide is removed, and pro-VWF forms C-terminal dimers. Upon transport to the Golgi apparatus (Golgi), the carbohydrates are processed into complex oligosaccharides, sulfation occurs, and C-terminal pro-VWF dimers are assembled into high-molecular-weight multimers. Before exiting the Golgi, VWFpp is proteolytically cleaved from VWF but remains noncovalently associated with the mature VWF multimers. Both VWFpp and mature VWF multimers are either constitutively secreted or routed to regulated secretory granules, Weibel–Palade bodies in endothelial cells, or α-granules in platelets. Once secreted into plasma through either the constitutive or regulated secretory pathway, VWFpp and mature VWF multimers cease to be noncovalently associated and circulate in plasma independently of one another. This research was originally published in *Blood* [78] © the American Society of Hematology. Adapted from [78].

Wagner *et al*., pro-VWF monomers accumulate in the ER. These results also indicate that glycosylation is required for dimerization to occur [84]. McKinnon and colleagues demonstrated that four N-linked glycan sites in VWFpp (N99) and mature VWF (N857, N2400, N2790) were found to be critical for the secretion of VWF [106]. In addition to glycosylation and dimerization, exit from the ER is also dependent upon the proper folding of the VWF protein. Misfolded proteins are selected in the ER and targeted for degradation, although many cells may retain misfolded proteins in the ER [107, 108]. A number of mutations have been identified in patients with VWD that result in impaired VWF secretion or ER degradation [109–112]. Defective VWF processing within the ER may contribute to the VWD phenotype observed in patients.

VWF processing in the Golgi

When pro-VWF dimers reach the Golgi, O-linked glycosylation occurs, glycans are trimmed, and galactose and sialic acid are added to form complex-type carbohydrates [103]. The O-linked glycome of VWF is comprised of 18 different glycan structures, including ABH antigens and disialosyl motifs [113]. In addition, the carboxyl-terminal pro-VWF dimers form amino terminal-linked multimers that may exceed 20 million in size (Figures 2.2 and 2.3). VWF multimerization will be discussed in more detail below. An additional modification that occurs in the Golgi is the proteolytic removal of the 741-amino acid VWFpp (Figure 2.2). VWFpp is believed to be cleaved from mature VWF by the enzyme furin. The cleavage of VWFpp appears to occur in the Golgi, as furin has been found to co-localize with other Golgi-resident proteins [114]. The site of propeptide cleavage is targeted by the sequence motif Arg-Xxx-Arg/Lys-Arg at the carboxyl-terminal end of the propeptide [115]. The furin-cleaved VWFpp remains associated with the mature VWF multimers until both proteins are eventually secreted from the cell [75, 78, 79, 82]. At pH 6.4, in the presence of calcium, mature VWF and VWFpp are associated, while at pH 7.4, this interaction is not sustained [76]. The conditions that promote association are similar to the pH and calcium levels found in the Golgi. In contrast, VWFpp and mature VWF circulate independently in plasma, which has an approximate pH of 7.4.

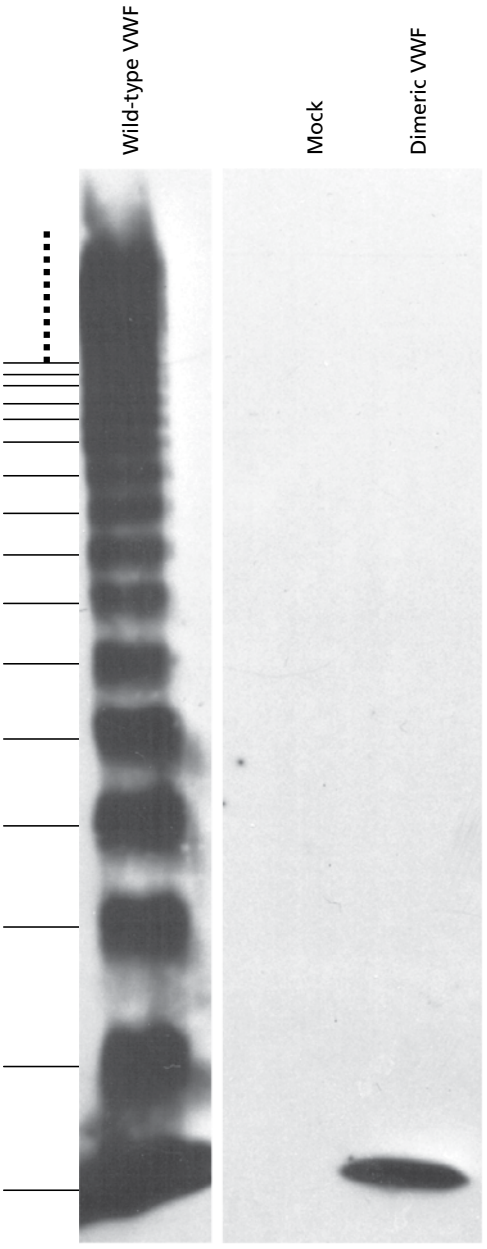

Figure 2.3 von Willebrand factor (VWF) forms high-molecular-weight multimers. VWF forms a C-terminal dimer in the endoplasmic reticulum, which is generally the smallest form of VWF secreted into plasma. In the Golgi, these C-terminal dimers form N-terminal disulfide bonds to create tetramers, hexamers, octomers, and other such high-molecular-weight oligomers. The multimeric structure of expressed VWF constructs was analyzed nonreduced on a 2% agarose-SDS gel. Expressed wild-type VWF (lane 2, "Wild-type VWF") shows a full range of multimers. The mock-transfected control is shown in lane 2 (Mock). Expression of propeptide-deleted VWF results in loss of multimerization, and only a dimeric VWF species is secreted (lane 3, "Dimeric VWF"). The highest-molecular-weight VWF multimers are the most active in platelet binding and clot formation. This research was originally published in *Blood*. [78] Haberichter *et al*. 2003/with permission from © Elsevier.

VWF multimerization

The unique process of VWF multimerization has been extensively investigated. The compartmentalization of VWF processing steps is best represented by these two distinct VWF polymerization steps. Dimerization of VWF has been shown to be a step that is independent of VWF multimerization [87, 96]. The carboxylterminal dimerization of VWF discussed above is accomplished in the ER, while amino-terminal multimerization of C-terminal VWF dimers is completed in the Golgi. The differential compartmentalization of these two processes implies that different enzymes or mechanisms regulate these two events. The ER is the most likely site for disulfide bond formation, where the neutral pH and necessary oxidoreductase enzymes, such as protein disulfide isomerase (PDI), promote the process of disulfide bonding. The acidic pH, together with the lack of chaperones and oxidoreductases in the Golgi, makes for a desolate environment for disulfide bond formation and rearrangement. However, VWF multimers are formed in a head-to-head orientation between adjacent D3 domains within C-terminal dimers to create oligomers that can exceed 20 million Da in size (Figure 2.3). ER to Golgi transport vesicles may not be able to accommodate the excessively large VWF multimers [86]. For this reason, VWF has evolved a unique mechanism to accomplish multimerization in the Golgi.

The critical role of VWFpp in the multimerization of VWF has been documented by a number of independent studies. Deletion of VWFpp does not affect the secretion of VWF from the cell, but the secreted VWF is in a dimeric form, and multimerization is completely abolished [87, 100, 101]. Multimerization of this dimeric VWFpp-deleted VWF can be restored by coexpression with VWFpp. Expression of VWFpp and VWFpp-deleted mature VWF as two separate expression plasmids results in the formation of a normal spectrum of VWF multimers. VWFpp does not need to be expressed as a contiguous protein with VWF (e.g., pro-VWF) to facilitate VWF multimerization. However, a study by Verweij and colleagues demonstrated that VWFpp cleavage from mature VWF is not a prerequisite for multimerization [116]. The introduction of a mutation that prevents furin cleavage, Arg763Gly, still permitted the formation of high-molecular-weight VWF multimers. VWF multimerization has also been demonstrated using VWF dimers in a cell-free system. A continued presence of an acidic pH was required for multimerization to proceed. However, in this cell-free system, multimerization required VWFpp to be a contiguous part of VWF. Only pro-VWF dimers formed high-molecular-weight multimers [83]. In sum, these studies indicate that VWFpp can facilitate multimerization of VWF dimers when expressed either as full-length VWF or as two separate proteins. VWFpp is absolutely required for VWF multimerization, whether expressed *in cis* or *in trans*.

Both the D1 and D2 domains that comprise VWFpp are necessary to accomplish VWF multimerization. Journet and coworkers demonstrated that the deletion of either domain results in the formation and secretion of VWF dimers [80]. The D domains in VWF are rich in cysteine residues and fairly

homologous, with an alignment of 23 cysteines between the four D domains [7, 13]. Both the D1 and D2 domains contain vicinal cysteine motifs, CXXC sequences (159-CGLC and 521-CGLC), that are similar to those found at the active site of disulfide isomerases. A study by Mayadas and Wagner showed that insertion of a glycine residue into either of these vicinal cysteine motifs results in the formation of dimers [117]. The dimeric VWF was efficiently transported to the Golgi and secreted from the cell, indicating that the insertion did not have a global structural effect but did abolish multimerization, highlighting the importance of the vicinal cysteines in VWF multimerization.

It has been proposed that VWF may overcome the limitations of the Golgi environment by using its own propeptide to facilitate multimerization by catalyzing disulfide exchange through the use of these vicinal cysteines. This mechanism would predict that VWFpp and the mature VWF subunit should form a transient disulfide bond in the cell before multimerization. Recently, this type of intermediate has been identified in the ER by expression studies using a recombinant D1–D2–D′–D3 truncated VWF protein and two-dimensional gel electrophoresis [118]. The disulfide-linked intermediate was found to rearrange in the Golgi, and both free VWFpp and D′–D3 dimers were secreted from the cell. This study provided support for the model in which VWFpp functions as an oxidoreductase to facilitate multimerization of VWF in the Golgi. In an extension of this study, two amino acids in the D3 domain of VWF were identified that were important in the multimerization process. Both Cys-1099 and Cys-1142 were found to be oxidized when VWF multimerization succeeds and reduced when it does not [93]. When either cysteine is mutated to an alanine, multimerization does not occur. The exact nature of how these cysteines form intersubunit disulfide bonds is unknown.

Defects in the multimerization of VWF are one cause of type 2A VWD in patients. Patients with type 2A VWD are characterized by decreased high-molecular-weight multimers and decreased platelet binding function [2]. The lack of high-molecular-weight multimers in these patients is the result of either increased degradation of plasma multimers by ADAMTS13 or defective multimerization and secretion from the cell. In the case of type 2A VWD caused by increased ADAMTS13 cleavage, the VWF undergoes normal multimerization within the cell but is degraded upon secretion into plasma. The majority of mutations in individuals with this type of VWD have been identified in the A2 domain [49]. Other type 2A VWD mutations cause defects in multimer formation within the cell. Mutations in these patients have been identified in both the D1 and D2 domains of VWFpp, the D3, and CK domains of mature VWF [52, 53, 55, 99, 119, 120]. Not surprisingly, these are the domains that are believed to play a significant role in multimer formation. Defective VWF processing within Golgi may contribute to the VWD phenotype observed in patients.

Regulated storage of VWF in Weibel–Palade bodies and α-granules

VWF is synthesized exclusively in endothelial cells and platelets produced by megakaryocytes and is stored for regulated release in Weibel–Palade bodies and α-granules (Figure 2.4). Regulated secretion allows for the rapid release of stored proteins at a local site to modulate coagulation, fibrinolysis, and inflammation. The Weibel–Palade body is a rod-shaped organelle (Figure 2.4) that is 1–2 μm in width and up to 4 μm in length [121]. These granules have longitudinal striations comprising closely packed tubules that are composed of VWF multimers [122]. Platelet α-granules appear to contain similar

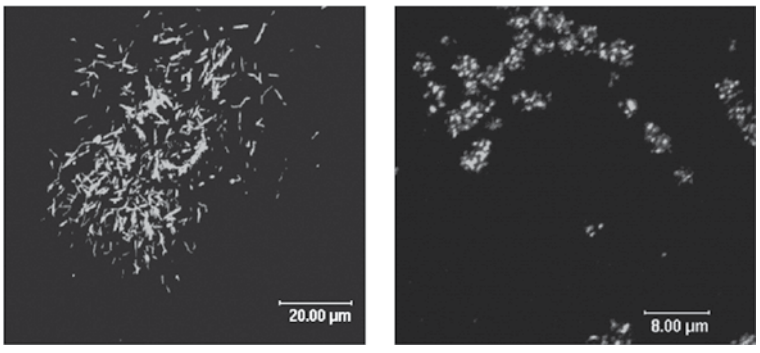

Figure 2.4 Regulated storage of von Willebrand factor (VWF) in endothelial cells and platelets produced by megakaryocytes. Endothelial cells and platelets store VWF in regulated secretory granules termed Weibel–Palade bodies and α-granules, respectively. (a) VWF in human umbilical vein endothelial cell Weibel–Palade bodies. Weibel–Palade bodies are rod-shaped granules up to 4 μm in length consisting of closely packed tubules of VWF. Human umbilical endothelial cells were cultured on slides, fixed, permeabilized, immunostained for VWF, and examined by confocal microscopy. VWF appears to be packaged in long rod-like Weibel–Palade bodies. (b) VWF in human platelet α-granules. Platelet α-granules also contain similar tubular structures, but the granules are not elongated or rod-like. Human platelets were spun on slides, fixed, permeabilized, immunostained for VWF, and examined by confocal microscopy. VWF is localized in α-granules that are much smaller than Weibel–Palade bodies. This research was originally published in *Blood*. [78] Haberichter *et al*. 2003/with permission from © Elsevier.

groups of tubular structures when examined by electron microscopy [123]. The VWF stored in Weibel–Palade bodies and α-granules contains some of the highest molecular weight multimers that are the most efficient for binding to extracellular matrix and to platelets to promote hemostasis. Endothelial cell Weibel–Palade bodies and platelet α-granules also contain the membrane protein P-selectin, among other proteins [124]. When Weibel–Palade bodies or α-granules undergo exocytosis, VWF is released and P-selectin is expressed on the surface within minutes of exposure to an agonist [125]. *In vivo,* exposure to physiologic stimuli including exercise and epinephrine or administration of DDAVP (1-deamino-8-D-arginine vasopressin) results in a rapid rise in plasma VWF levels, presumably owing to exocytosis from Weibel–Palade bodies [126]. The mechanisms controlling the trafficking of VWF to the regulated storage pathway have been extensively investigated. While some studies have examined the storage of VWF in α-granules, the majority of studies on VWF-regulated storage have utilized cultured endothelial cells or transfected mammalian cells.

In addition to VWF, VWFpp is also stored in endothelial cell Weibel–Palade bodies and platelet α-granules [127]. In Weibel–Palade bodies, mature VWF and VWFpp are found in a 1:1 molar ratio [75]. When expressed in a variety of mammalian cells, including AtT-20 mouse pituitary cells, CV-1 monkey kidney cells, MDCK cells, and RIN5F rat insulinoma cells, VWF is trafficked to storage granules [79, 82, 85, 128]. Expression studies utilizing transfected mammalian cells have shown that VWFpp is required for the targeting of VWF to storage granules [82]. Expression of mature VWF lacking its propeptide results in the loss of VWF trafficking to storage granules [79, 82, 85]. Coexpression of mature VWF *in trans* with VWFpp, as two separate expression plasmids, results in the restoration of granular trafficking of VWF, indicating that VWF granular storage does not require VWFpp to be a contiguous part of VWF. Both of the D domains have been reported by Journet and coworkers to be necessary for granular trafficking to storage, and deletion of either domain results in the loss of VWF granules [80].

Several lines of evidence indicate that VWFpp plays an active role in trafficking VWF to the regulated storage pathway. Propeptide-deleted mature VWF (Δpro) expressed alone in AtT-20 cells does not traffic to granules but instead demonstrates diffuse staining, indicative of ER localization (Figure 2.5). In contrast, VWFpp independently traffics to endogenous ACTH-containing storage granules in AtT-20 cells (Figure 2.5) and to Weibel–Palade bodies in endothelial cells [79, 129]. When VWFpp was covalently linked to an unrelated, nonsecretory granule protein, C3α, and expressed in bovine aortic endothelial cells, both VWFpp and C3α were trafficked to Weibel–Palade bodies, where they underwent regulated release [129]. These studies indicate that VWFpp contains the necessary signal, either a linear sequence or conformation, for sorting to the regulated secretory pathway. When a series of C-terminal and N-terminal truncations of VWFpp were expressed in AtT-20 cells, a truncated VWFpp comprising amino acids 387–545 was found to be co-localized in storage granules with endogenous ACTH [130]. The putative sorting signal may be located in the first 140 amino acids of the D2 domain of VWFpp.

When VWFpp is expressed *in trans* with mature VWF, both proteins are stored in granules (Figure 2.5). Studies suggest that VWFpp functions as an intracellular chaperone: VWFpp contains the signal for sorting granules and co-traffic mature VWF. In this model, VWFpp and mature VWF remain associated in the Golgi and traffic together to the granule. The low pH and calcium concentration that is found in the Golgi promote this association, while at a plasma pH of 7.4, this association is not maintained [76]. This model requires a site within VWFpp for association with VWF and, conversely, a site within mature VWF for interaction with VWFpp. By using

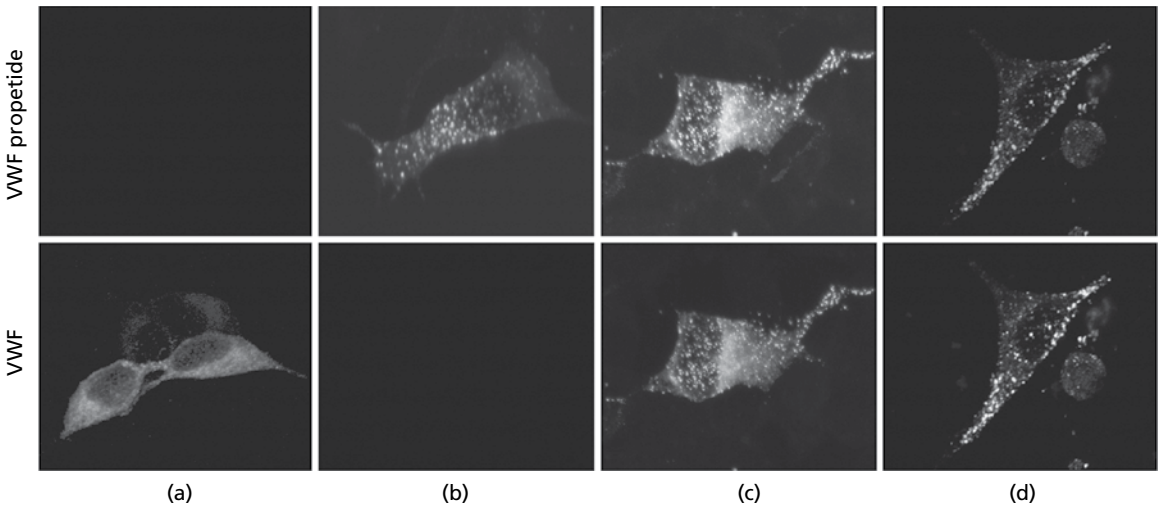

Figure 2.5 Expression of VWF in AtT-20 cells. (a) Propeptide-deleted VWF is not sorted to granules, (b) propeptide alone sorts to granules, (c) propeptide co-expressed with peptide-deleted VWF sorts to granules, (d) full-length VWF sorts to granules.

a series of human/canine chimeric VWFpp and mature VWF expression plasmids, and exploiting the differential interspecies storage difference, potential interaction sites have been identified [78]. Amino acid 416 in the D2 domain of VWF and amino acid 869 in the D3 domain of mature VWF were found to be critical for the noncovalent interaction and subsequent storage of VWF. It is not clear whether these two amino acids directly interact or enable a conformation that promotes association. It is very likely that other amino acids are involved in the association of VWFpp and VWF.

Formation of the VWF multimer precedes the formation of Weibel–Palade bodies [76]. However, a number of studies have confirmed that VWF multimerization is not a prerequisite for VWF granular storage. Wagner and colleagues demonstrated that the deletion of a portion of the C-terminal VWF sequence resulted in a dimeric VWF species that was stored in granules [82]. Disruption of either of the vicinal cysteine motifs in VWFpp resulted in a loss of multimerization with normal granular storage [117]. In a study utilizing human/canine chimeric VWFpp and mature VWF expression plasmids, VWF multimerization did not correlate with VWF granular storage: some fully multimerized VWF proteins were not stored in granules, while other nonmultimerized VWF proteins were found to be stored in granules [78]. A number of expression studies have been completed characterizing the effects of mutations identified in patients with VWD on VWF multimerization and granular storage. Several mutations, including Y87S, R273W, C788R, C1157S, C1225G, and C1234W, were found to cause defects in multimerization, yet VWF granular storage was maintained [77, 112, 119, 131]. These studies suggest that the regions within VWFpp that facilitate VWF multimerization are different from the sites regulating VWF granular storage.

Both multimerization and VWFpp cleavage are thought to be accomplished in the Golgi before the formation of storage granules [79, 82]. Two different VWF mutations causing a lack of VWFpp cleavage have been characterized, an Arg763Gly mutation and a splice site mutation causing loss of the furin cleavage site [85, 132]. Both variants were trafficked to storage granules, demonstrating that cleavage of VWFpp from mature VWF does not appear to be a prerequisite for granular trafficking.

Weibel–Palade body biogenesis

Expression studies of VWF have utilized several different cell lines, including AtT-20, RIN5F, CV-1, MDCK, HEK-293, COS-1, 3T3, and CHO cells. It was not surprising that cell lines containing endogenous secretory granules, such as AtT-20 and RIN5F cells, were able to traffic VWF into granules and that many cell lines not thought to contain secretory granules, such as COS-1 and CHO cells, did not form VWF-containing granules. However, unexpectedly, expression of VWF induced the formation of VWF-containing granules in HEK293 and MDCK cells, which are not thought to have regulated secretory granules [112, 128]. Additionally, when VWF is expressed in AtT-20 cells, the VWF-granules that are formed do not co-localize with endogenous ACTH-containing granules but instead form granules that only contain VWF [133]. These studies indicate that VWF has the ability to induce the formation of its own storage granule.

The biogenesis of the Weibel–Palade body appears to be a VWF-driven event [77, 122]. Weibel–Palade bodies do not exist in the absence of VWF and cannot be detected in endothelial cells harvested from VWF-deficient dogs (Figure 2.6) and VWF knockout mice [77, 134, 135]. Neither VWF nor VWFpp expression can be detected in VWD canine aortic endothelial cells (Figure 2.6). In these VWF-null cells, the Weibel–Palade body membrane protein, P-selectin, is redirected to lysosomes. Expression of full-length VWF in VWF-deficient canine endothelial cells (Figure 2.6) and T-24/ECV304 cells resulted in the formation of VWF-containing granules that were morphologically similar to Weibel–Palade bodies [77, 136]. In each of these studies, endogenously synthesized P-selectin was redirected from lysosomes to the VWF-containing granule, validating the formation of a Weibel–Palade body. Other expression studies in AtT-20 cells and HEK293 cells have shown that coexpression of VWF and P-selectin results in collocalized granular storage of the two proteins [112, 133]. P-selectin was later found to bind to the D′–D3 domains of VWF [137]. Together, these studies confirm the role of VWF in the biogenesis of the Weibel–Palade body.

Further studies utilizing VWF-deficient endothelial cells from dogs with type 3 VWD demonstrated that both VWFpp and mature VWF were required for the biogenesis of Weibel–Palade bodies [77]. Habericher *et al*. demonstrated that neither VWFpp nor mature VWF were sufficient for granule formation when expressed independently. By expressing a dimeric VWF variant, Y87S, Haberichter *et al*. also demonstrated that multimerization was not required for Weibel–Palade body biogenesis, as this variant formed granules that recruited P-selectin. This study suggests that VWFpp may provide the "sorting signal" and mature VWF provides the core of aggregation for granule budding. Expression studies using C-terminal truncations of VWF demonstrated that VWFpp and the D–D3–A1 domains of mature VWF were required to store VWF as tubules that correlate with the elongation of Weibel–Palade bodies [127]. The VWF tubule formation and elongation were both found to be dependent on an acidic pH level. Furthermore, these studies strongly suggested a direct role for VWFpp in compacting VWF into tubules. Most recently, VWF tubule assembly has been recreated in an *in vitro* study using only purified VWFpp and D′–D3 domains of VWF [102]. Assembly of tubules from these two proteins was found to be dependent upon a low pH and the presence of calcium. A three-dimensional reconstruction of electron microscopy images demonstrated tubules containing a repeating unit of one D′–D3 dimer and two VWFpp molecules. These studies have narrowed the requirements for Weibel–Palade body biogenesis to the D1–D3 domains of VWF. The requirements for VWF trafficking to α-granules in platelets may be similar to those for Weibel–Palade body trafficking, although mechanisms may not be identical since α-granules are still formed in the absence of VWF.