Journal of Thrombosis and Thrombolysis 1999;8:197–205

Recombinant von Willebrand Factor: Potential Therapeutic Use

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Abstract. **Human von Willebrand factor (vWF) produced by recombinant technology offers a new perspective in treatment of von Willebrand disease (vWD). Several limitations connected with plasma-derived vWF concentrates, such as proteolytic degradation during the manufacture process, variation in multimer composition, lack of high molecular weight multimers, and donor dependence, can be overcome by rec-vWF. Recombinant vWF (rec-vWF) is produced by continuous fermentation of transformed mammalian cells. Biotechnological processes have been developed to isolated rec-vWF fractions with low, medium, and high degrees of multimerization. Structural analysis of rec-vWF demonstrated that it undergoes post-translational modifications comparable with plasma-derived vWF, such as multimerization, pro-peptide processing, and glycosylation. Functional analysis showed that rec-vWF exhibited activities comparable with plasma-derived vWF, such as platelet binding, platelet aggregation, collagen binding, and coagulation factor VIII (FVIII) binding. Collagen binding and platelet aggregation activity increased with the increasing multimer size of rec-vWF. Infusion of rec-vWF in antibody-induced vWF-deficient mice resulted in a significant decrease in bleeding. Infusion of rec-vWF in vWF-deficient dogs and pigs with severe vWD caused an increase in the endodenous FVIII level. Stabilization of FVIII in vivo was mediated both by high and low molecular weight rec-vWF molecules. Apparently, rec-vWF resisted proteolytic degradation in the circulation and no satellite bands were formed. Functional analysis *in vitro* and *in vivo* demonstrated the therapeutic potentials of rec-vWF, correction of vWF level, and stabilization of FVIII in plasma.**

Key Words. **von Willebrand factor, recombinant, bleeding, collagen binding, platelet aggregation**

*V*on Willebrand disease (vWD) is the most common bleeding disorder in humans [1,2]. It is caused by inherited quantitative or qualitative defects in von Willebrand factor (vWF). vWF is an adhesive plasma glycoprotein synthesized in vascular endothelial cells and megakaryocytes. vWF circulates in human plasma as a series of multimers ranging in size from about 450 kD to more than 20,000 kD [3–6]. Once released, vWF serves a dual purpose in hemostasis. It is an adhesive protein that acts as a bridge between platelet glycoproteins and vascular subendothelium. In addition, vWF binds coagulation factor VIII (FVIII) and circulates with it as a noncovalently linked complex. In this manner, vWF plays a key role in hemostasis by initiating platelet adhesion at the sites of vascular injury and by localizing FVIII to sites where it can participate in the generation of thrombin and fibrin clot [7].

The manifestations of vWD demonstrate the pathophysiological significance of the different biological functions of this protein. The disease is characterized by prolonged bleeding time, defective platelet adhesion, and an accompanying deficiency in FVIII. The most severe form of vWD is type 3, which has a prevalence of one to three cases per million and is an autosomal recessive disorder in which vWF is virtually absent. It is caused by deletion, frameshift, and nonsense mutations. The most common form of the disease is type 1, in which there is a partial quantitative deficiency of vWF, with an estimated prevalence of approximately 100 per million [1,2,7]. vWD type 1 is always accompanied by a parallel decrease in FVIII procoagulant activity. vWD type 2A refers to qualitative variants with decreased platelet-depended function associated with the loss of high molecular weight vWF multimers. By contrast, vWD type 2B refers to qualitative variants with increased affinity for platelet glycoprotein Ib. Qualitative variants with decreased platelet-dependent function that is not caused by the absence of high molecular weight vWF multimers are known as vWD type 2M.

The treatment of mild vWD involves raising the endogenous level of vWF by administration of the synthetic vasopressin analogue DDAVP (1-desamino-8-D-arginine vasopressin). In severe vWD, currently human plasma-derived concentrates are used in replacement therapy [7,8]. However, those concentrates contain both FVIII and vWF. Apparently, due to the nature of their preparation and their sensitivity of vWF and FVIII to proteolytic degradation, FVIII/vWF concentrates are produced from human plasma or cryoprecipitate, and there is a great variation in their purity, structural intactness, and the

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Received 16 June 1998; accepted in revised form 7 October 1998

molecule ratio of FVIII and vWF in different commercial preparation [9].

The field of biotechnology has already exerted a major impact in the management of cardiovascular and bleeding disorders and has been applied for replacement therapy in transfusion medicine via the development of recombinant drugs, such as tissue plasminogen activator, urokinase, platelet targeting antibodies, hirudin, coagulation factors VIII and VIIa, and human albumin [10–12]. Although enormous progress has been made in the purification of plasma proteins, the need to avoid the risks of transmitting viral infections has speed up the development of further recombinant therapeutic preparations. The biotechnological and preclinical results of a recombinant human vWF (rec-vWF) preparation are summarized here.

Biosynthesis and Structure of Human vWF

The precursor polypeptide produced in endothelial cells, pre-pro-vWF, consists of a 22-residue signal peptide, a 741-residue pro-peptide, and a 2050-residue polypeptide found in mature plasma vWF (Figure 1). After removal of the signal peptide, the resulting pro-vWF subunits engage in a complex biosynthetic process thought to begin with the formation of a dimer containing two pro-vWF subunits linked through disulfide bonds. The protomeric units of the multimeric series are then assembled into higher order multimers through disulfide bonding of dimers. The vWF pro-peptide is cleaved from the multimeric vWF before it is released into the circulation (Figure 2) [3,5,13,14]. During its biosynthesis, vWF is exposed to several post-translational modifications (see Figure 1). Concurrent with the formation of multimeric vWF, the mature subunit is modified with the addition of 12 N-linked and 10 O-linked carbohydrate chains. Specific N-linked carbohydrate structures are additionally modified by sulfatation [16]. vWF produced by endothelial cells can follow two pathway of secretion, namely, a constitutive pathway directly linked to synthesis and a regulated pathway involving storage of mature molecules in appropriate granules, in the Weibel-Palade body, and release after stimulation. The vWF stored within Weibel-Palade bodies is composed of the largest multimeric species, whereas it is generally thought that the vWF processed through the constitutive pathway does not multimerize to the same extent [3–5].

The multimeric pattern of vWF consists of regularly spaced bands that can be visualized by sodium dodecyl sulfate (SDS)-agarose gel electrophoresis (Figure 3). However, analysis of vWF by high-resolution SDS-agarose electrophoresis has shown that each multimer in plasma consists of three major bands, described as triplet structures. These are composed of an intermediate (central) band, a slower migrating band (upper band), and a faster migrating band (lower band). The appearance of low molecular weight forms of vWF and of triplet structures has been ascribed to proteolytic degradation of mature vWF [6,10,16–19]. A proteolytic cleavage site at the position Tyr842-Met843 of the mature vWF that leads to a N-terminal fragment containing 842 amino acids and a C-terminal fragment containing 1208 amino acids has been identified [16]. Recently a protease has been isolated from human plasma cleaving vWF into fragments at Tyr842-Met843 [19].

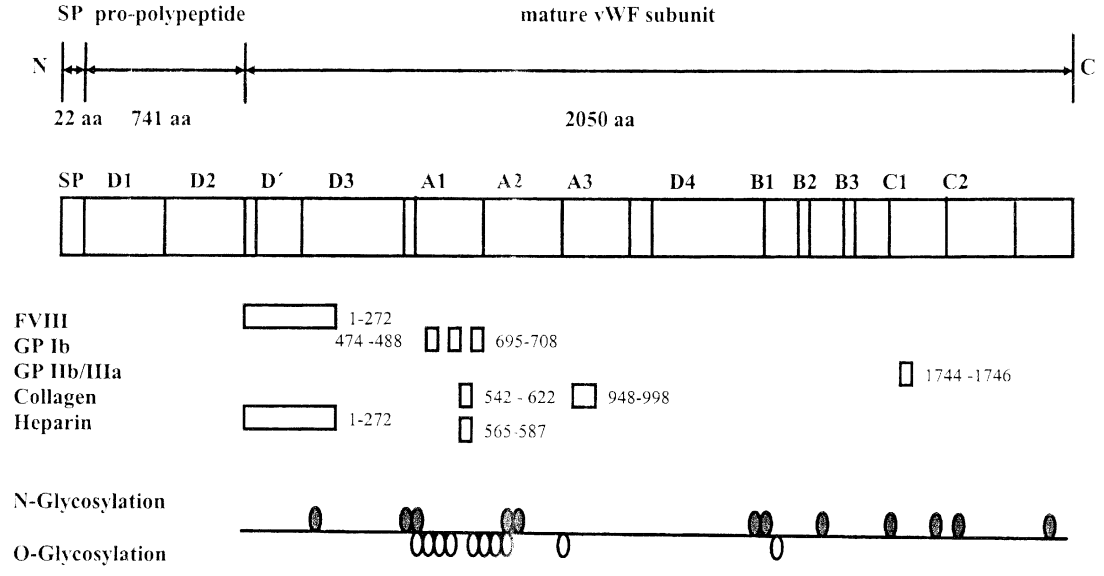

Fig. 1. *Schematic representation of pre-pro-vWF subunit. Structural and functional domains and glycosylation sites of the mature vWF subunit are indicated.*

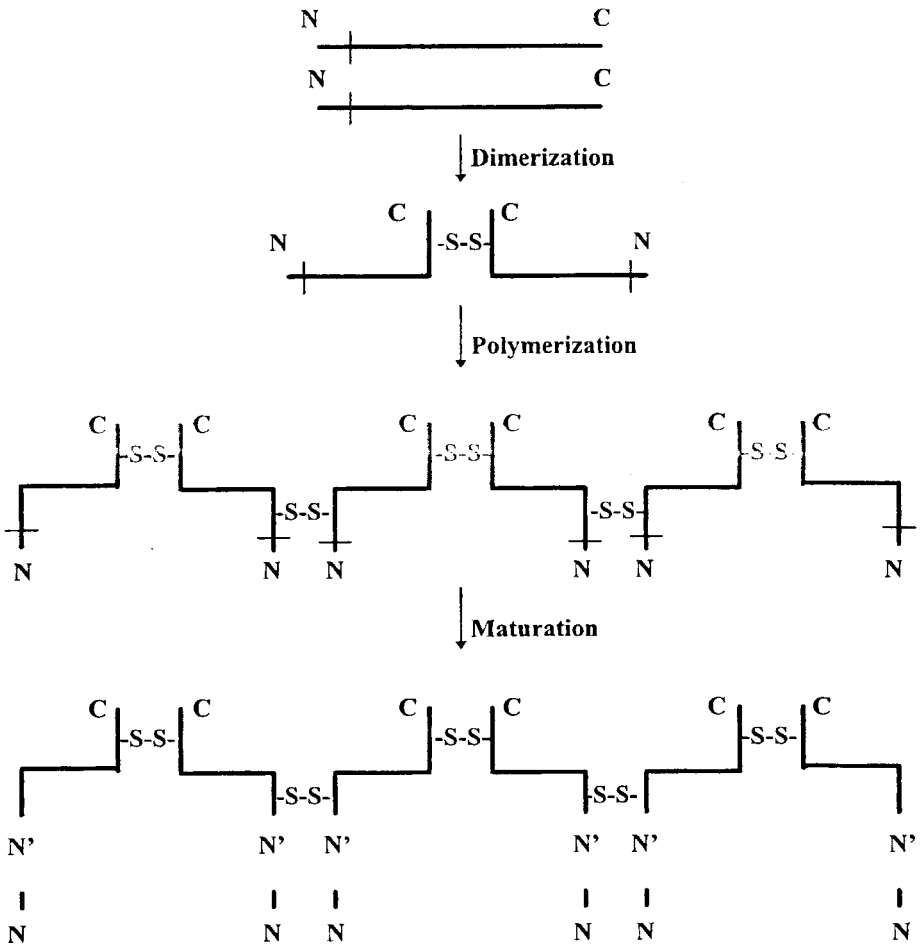

Fig. 2. *Assembly of vWF multimers. First, two identical pro-vWF polypeptides form disulfide-linked dimers. Second, pro-vWF-dimers are linked through disulfide bonds at their N-termini and large pro-vWF multimers are formed. Before release, the pro-peptide sequence is cleaved from mature vWF multimers.*

Production of Recombinant vWF

vWF was cloned in 1986 [20], and rec-vWF and several of its fragments were expressed in mammalian cells [21–43], prokaryotic cells [44,45], and insect cells [46]. However, rec-vWF and rec-vWF fragments were produced mainly at laboratory scale to study the molecular structure, to detect receptor binding sites and modified regions, as well as to interpret the post-translational processing and multimerization of the molecule.

For permanent expression and production of rec-vWF at a preparative scale, Chinese hamster ovary (CHO) cells were chosen as host cells [47–51]. CHO cells expressing recombinant pre-pro-vWF were established using the calcium phosphate transfection technique and screening for a high-level producer [47,50]. For scale up CHO cells were grown in roller bottles, and for preparative scale fermentation they were cultivated in a high cell density perfusion bioreactor using microcarriers for cell attachment [48,51]. During the production phase, fermentation medium was not supplemented with serum. rec-vWF was expressed permanently and secreted into the fermentation medium for several weeks [48] (Figure 4). Purified rec-vWF is obtained from fermentation medium by centrifugation, ultrafiltration, and a combination of ion exchange and affinity chromatography [50–53]. Both heparin affinity chromatography [53] and cation exchange chromatography [54] were exploited to separate low, medium, and high molecular weight multimers of rec-vWF (Figure 5).

Structural characterization of rec-vWF

rec-vWF expressed by serum-free fermentation of CHO cells exhibited a multimeric pattern similar to human plasma–derived vWF comprising multimers with a low, medium, and high degree of multimeriza-

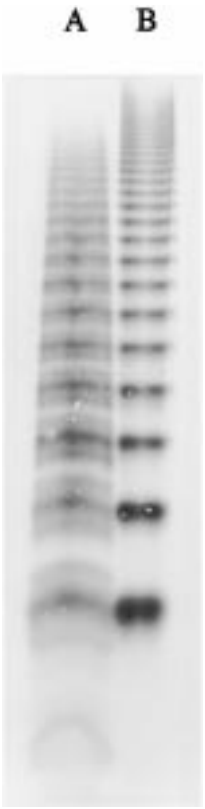

Fig. 3. *Multimer structure of human plasma vWF (*A*) and rec-vWF (*B*) analyzed by SDS-2% agarose gel electrophoresis. (From Fischer et al. [52], with permission of Portland Press.)*

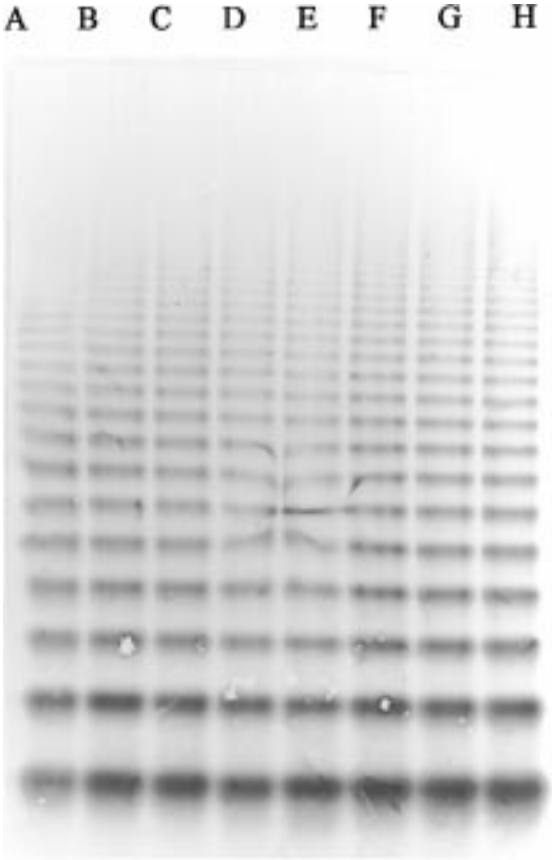

Fig. 4. *Multimer analysis of rec-vWF during adaptation of CHO cells to serum-free medium.* A: *rec-vWF produced at day 18 in fermentation medium containing 2.5% serum.* B: *rec-vWF produced at day 24 during adaptation to serum-free medium.* C: *rec-vWF produced at day 29 during adaptation to serum-free medium.* D: *rec-vWF produced at day 35 in serum-free medium.* E: *rec-vWF produced at day 44 in serum-free medium.* F: *rec-vWF produced at day 51 in serum-free medium.* G: *rec-vWF produced at day 56 in serum-free medium.* H: *rec-vWF produced at day 65 in serum-free medium. (From Fischer et al. [48] with permission of Elsevier Science.)*

tion of vWF subunits. However, structural analysis revealed that a significant proportion of rec-vWF was not completely processed and still contained covalently attached pro-peptide [47,50]. Homo-dimers and hetero-dimers and hetero-polymers were detected by two-dimensional agarose gel electrophoresis [47,50]. Similar observation have been made in studies by other research groups during expression of rec-vWF in heterologous cells [24–29,41,55–57]. In contrast to human endothelial cells, apparently most heterologous cells lack basic requirements for sufficient pro-peptide cleavage, such as a vWF-specific pro-peptide cleaving endoproteinasease.

Due to the significant role of the pro-peptide in the multimerization process of vWF [24,25,27], expression of rec-vWF without pro-peptide was not possible. To produce rec-vWF free of pro-peptide, the mammalian endoproteinase furin was subsequently and additionally coexpressed in CHO cells [48,49,51,58]. Furin is known to mediate precursor processing of proteins in the constitutive secretory pathway [59] during their passage through the trans-Golgi network [60–62]. Co-expression of furin in the CHO-rec-vWF system resulted in completely processed rec-vWF lacking pro-peptide [48,49,51,52]. Two-dimensional agarose gel electrophoresis [48,52] demonstrated that all the multimers of rec-vWF contained exclusively mature subunits (Figure 6). Human plasma vWF is exposed to limited proteolytic degradation. Hydrolysis of the peptide bond Tyr842-Met843 by a specific proteinase results in both degradation of high molecular weight vWF and formation of vWF degradation products [16–19]. This proteolytic degradation is indicated by the occurrence of the triplet pattern, made visible by high resolution agarose gel electrophoresis [3–5,18,52]. Comparison of vWF with rec-vWF demonstrated that the intermediate (central) band of each triplet represents multiple numbers of vWF dimers [52]. The faster and slower migrating bands of the triplet have too

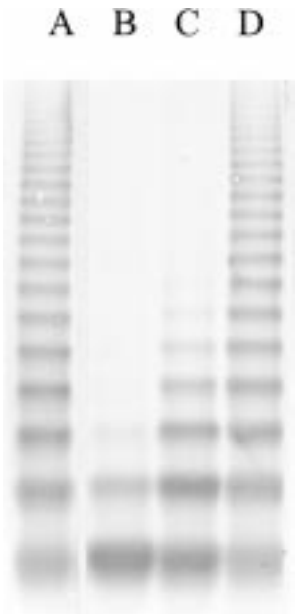

Fig. 5. *SDS-2% agarose gel electrophoresis of* (A) *rec-vWF,* (B) *low molecular weight rec-vWF multimers,* (C) *medium molecular weight rec-vWF multimers, and* (D) *high molecular weight rec-vWF multimers. (From Fischer et al. [51] with permission of Birkhäuser Publishing Ltd.)*

many or too few subunit structures. Obviously, this vWF-specific proteinase is not produced by CHO cells during fermentation. Thus, rec-vWF is not exposed to a proteolytic degradation process and no triplet structures are formed (see Figure 3). This makes the most obvious structural difference between human plasma vWF and rec-vWF.

Human vWF is a highly glycosylated plasma protein. vWF exhibits 12 N- and 10 O-glycosylation sites. Glycosylation contribute to physiological activity, receptor binding, clearance from the circulation, and structural stability. Carbohydrate analysis demonstrated that rec-vWF is glycosylated during its expression in CHO cells [50,51]. Carbohydrates attached to both N-glycosylation and O-glycosylation sites were detected. Similar to human plasma vWF, carbohydrate chains were composed of sialic acid, galactose, glucose, and mannose residues. Carbohydrate chains were part of the structure of rec-vWF and were not easily removed without denaturation of the molecule [51]. Removal of glycosylation significantly reduced the functionality of rec-vWF [50]. However, similar to other recombinant coagulation factors produced by CHO cells [63], rec-vWF contained terminal sialic acid-α(2-3)-galactose structures, whereas human plasma vWF contained sialic acid-α(2-6)-galactose units [51].

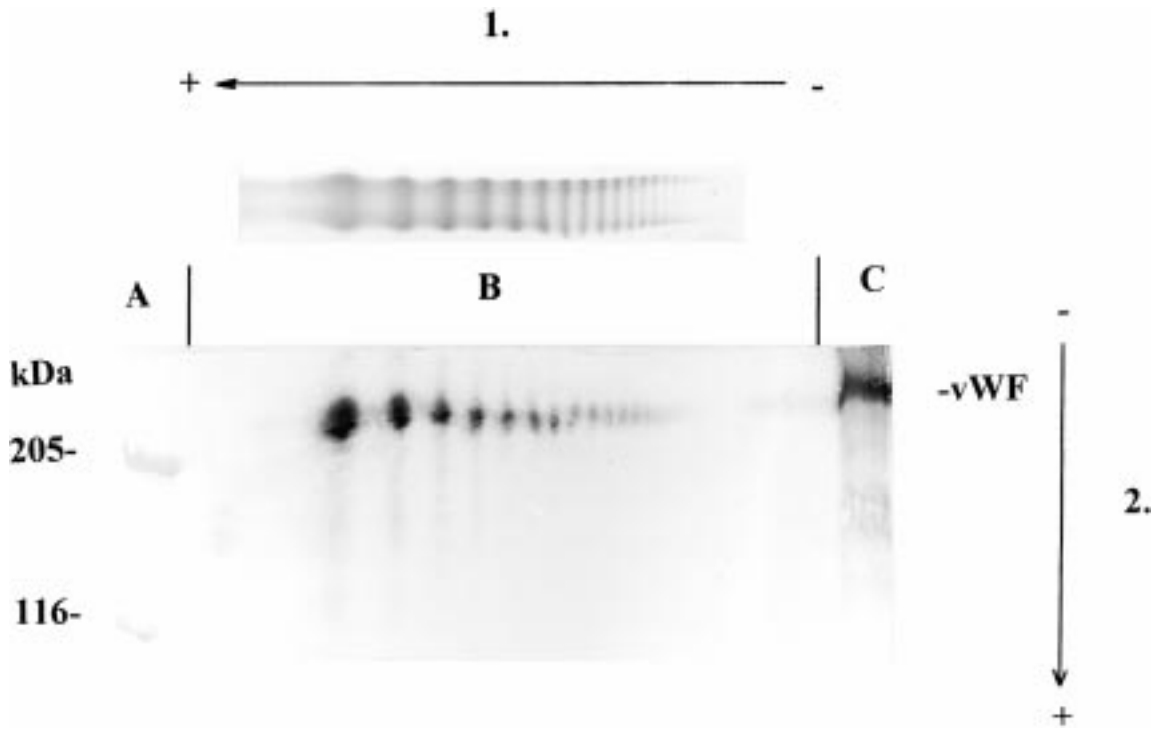

Fig. 6. *Two-dimensional analysis of multimer pattern of rec-vWF. rec-vWF was separated in the first dimension by nonreducing SDS-1% agarose gel electrophoresis. An individual gel lane containing the multimer pattern was cut out, incubated in reducing buffer, mounted on top of a 5% acrylamide gel, and electrophoresed in the second dimension* (B). *Simultaneously, denatured-reduced rec-vWF* (C) *and prestained reference proteins* (A) *were analyzed. (From Fischer et al. [48] with permission of Elsevier Science.)*

Functional Analysis of Recombinant vWF

Multiple interactions of repeating binding sites in vWF multimers with adhesive proteins of the subendothelium, such as collagen, and with receptors on the platelets, such as glycoprotein Ib (GP Ib) and the glycoprotein IIb/IIIa (GP IIb/IIIa) complex, lead to platelet aggregation and binding of platelets to the exposed subendothelium. Binding sites for collagen and GP Ib and GP IIb/IIIa area contained in each vWF subunit (see Figure 1) [3,5,64]. Binding of rec-vWF to platelets in the presence of ristocetin was dose dependent and reached saturation at high concentrations of rec-vWF [51]. Agarose gel electrophoresis demonstrated that binding of rec-vWF to platelets did not depend on the degree of multimerization; thus glycoprotein binding sites were functional active in all subunits of rec-vWF [51]. As *in vitro* laboratory methods [65], ristocetin cofactor activity (vWF:RistCof, measured by ristocetin-induced platelet aggregation) and collagen binding activity (vWF:CBA, analyzed by ELISA using collagen-coated microtitration plates) were used to characterize the functionality of rec-vWF. Because vWF functions to connect two or more platelets and to build a bridge between subendothelial collagen and platelets, a sufficient length of the multimeric molecule is essential. Thus, the larger multimers are being considered to be hemostatically more active [10,66]. Functional analysis demonstrated that vWF:RistCof and vWF:CBA increased with the increasing degree of multimerization of rec-vWF [51,53]. Platelet aggregation activity and collagen binding activity were 63 times and 350 times higher, respectively, in high molecular weight rec-vWF compared with low molecular weight rec-vWF [51,53]. rec-vWF-dimers virtually exhibited no vWF:RistCof and vWF:CBA [53]. The functional activity of rec-vWF corresponded to human plasma vWF. The interaction of low, medium, and high molecular weight rec-vWF with FVIII resulted in association rates of 3.0–3.9 $\times$ 10^6 M^{-1} s^{-1}, dissociation rates of 1.2–1.8 $\times$ 10^{-3} s^{-1}, and equilibrium binding constants of 2.2–2.5 $\times$ 10^9 M^{-1} [51, 53]. Apparently, binding of FVIII is less affected by the degree of multimerization of rec-vWF. Binding constants obtained for rec-vWF were equivalent to those of human plasma vWF [53,67,68].

rec-vWF was tested in three animal model *in vivo*: mice, dogs, and pigs [69–71]. rec-vWF at doses of 20–400 vWF:RistCof U/kg was injected into *in vivo* antibody-induced vWF-deficient mice [69]. Cuts were made at the tip of the tail, and bleeding time and intensity were determined. Infusion of 200 vWF:RistCof U/kg significantly decreased the rate of blood flow. However, bleeding stopped completely only when FVIII was also added. Injection of rec-vWF into vWF-deficient mice resulted in restoration of vWF multimers [69]. The effect of rec-vWF on hemostatic parameters and bleeding was investigated in vWF-deficient Dutch Kooiker dogs [70]. Infusion of rec-vWF was administered at doses of 30–75 vWF:RistCof U/kg. The half-life of vWF:Ag was substantially longer after administration of rec-vWF (<20 hours) compared with plasma vWF (8 hours). *In vivo* recoveries of vWF:Ag and vWF:RistCof were <59% and <78%, respectively. Infusion of rec-vWF into vWF-deficient dogs caused a substantial increase in the endogenous FVIII level, which was maintained even when vWF:Ag had decreased to an almost undetectable level.

After infusion of rec-vWF, FVIII activity was approximately two times the normal canine levels, peaking at 24–48 hours and remaining at this level for at least a further 24 hours. The half-life of FVIII in dogs did not depend on the survival of all of the multimeric species. Most interestingly, infusion of low molecular weight rec-vWF caused an increase in the endogenous FVIII plasma level similar to that seen after the infusion of rec-vWF containing the full range of multimers. Apparently, multimerization does not affect the binding of FVIII, or is the protective effect of vWF on FVIII related to the size of the multimers. Multimers of rec-vWF were gradually removed from the circulation. No formation of satellite bands was observed. High molecular weight multimers were removed from the circulation faster than low molecular weight multimers. Pigs with severe vWD received rec-vWF at doses of 35 and 70 vWF:RistCof U/kg [71]. The half-life of vWF:Ag was approximately 30 hours. After infusion of rec-vWF (35 vWF:RistCof U/kg), endogenous FVIII level slowly increased to near normal within 24 hours and remained at this level for more than 48 hours. Multimer analysis of the rec-vWF molecule after administration showed that the highest molecular weight multimers were the first to disappear from the circulation. The rec-vWF multimers showed a high degree of molecular integrity without the satellite bands that would have resulted from proteolytic degradation. The structure of rec-vWF multimers did not change after infusion.

In summary, *in vitro* tests demonstrated that rec-vWF exhibited functional properties comparable with plasma-derived vWF. Platelet aggregation and collagen binding were strictly dependent on the degree of multimerization. By contrast, low, medium, and high molecular weight multimers bound to FVIII with similar rates and binding constants. These results were confirmed by studies in mice, dogs, and pigs. Infusion of rec-vWF resulted in a decrease in bleeding, and in stabilization and on increase in the FVIII level.

Therapeutic Use of Recombinant vWF

vWD is a congenital bleeding disorder characterized by a complex hemostatic defect. Abnormal platelet function, expressed by a prolonged bleeding time, is a consistent finding and may be accompanied by de-

creased coagulation factor VIII procoagulant activity (FVIII:C). When present, the decreased FVIII:C is attributable to the reduced concentration of vWF [72]. In the treatment of vWD the aim is to correct the vWF-dependent defect in primary hemostasis and the factor VIII deficiency. The goals of therapy are to increase FVIII:C to the hemostatic level and to increase vWF to improve ristocetin cofactor activity and correct the prolonged bleeding time [73,74]. In types 1 and 2 (except 2B) vWD, this can be achieved by administration of DDAVP, which results in the release of FVIII and vWF into the circulation from biosynthetic stores. By contrast, moderate to severe hemophilia and type 3 vWD are currently treated by replacement therapy [75]. Plasma and cryoprecipitate have been the mainstay of the therapy for vWD for many years [76]. These blood products contain adequate amounts of FVIII:C and functional vWF to correct both defects. However, the vWF level of cryoprecipitate is dependent on the donor's pre-existing vWF level. FVIII and vWF copurify using many standard biochemical techniques. As a result, FVIII concentrates with an intermediate level of purity contain large amounts of vWF. However, there is frequently proteolytic degradation of the vWF during the purification of FVIII [9]. FVIII concentrates that contain significant amounts of vWF vary significantly from manufacturer to manufacturer in the relative sparing of the high molecular weight multimers from degradation [9]. However, as noted previously, the function of vWF is dependent on the normal vWF multimeric structure [7]. By contrast, highly purified commercial FVIII concentrates, used for many years in the treatment of hemophilia A, have been found to be ineffective for correcting the bleeding time in vWD, although they do bring FVIII:C activity up to normal [7,74].

Von Willebrand factor produced by recombinant technology offers a new perspective on the treatment of vWD. Several limitations connected with plasma-derived vWF concentrates, such as proteolytic degradation during the manufacturing process, variation in multimer composition, lack of high molecular weight multimers, and donor dependence, can be overcome by the rec-vWF. The recombinant molecule is produced by continuous fermentation of transformed cells, starting from a single cell clone. This enables one to obtain rec-vWF with constant functional and molecular properties during each production process. rec-vWF is not exposed to uncontrolled proteolytic degradation by plasma proteases, and the risk of virus transmission is virtually eliminated. Due to its production by mammalian cells, rec-vWF undergoes post-translational modifications, such as multimerization, pro-peptide processing, and N- and O-glycosylation. Both extensive structural and functional analysis *in vitro* and *in vivo* studies in animal models demonstrated that rec-vWF exhibits functionalities that make it comparable with human plasma-derived vWF. Purification conditions have been developed to either isolate the full range of rec-vWF multimers or to separate low, medium, and high molecular weight multimers [50,51,53]. This would provide the advantage of making available different products for different clinical applications. While the full range of vWF multimers is missing in type 3 vWD, high molecular weight multimers are missing in type 2A vWD and sometimes in type 2B vWD. Thus, replacement of plasma vWF should depend on which multimeric fraction of the molecule is missing. Because low molecular weight rec-vWF binds and stabilizes FVIII as effectively as high molecular weight multimers, such a preparation can be used to raise the endogenous FVIII level if necessary. In addition, low molecular weight rec-vWF may be used to stabilize FVIII preparations *in vitro*, for example, in recombinant FVIII preparations, without adding platelet aggregation activity.

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