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Review

Insights from von Willebrand disease animal models

C. V. Denis and D. D. Wagner*

The Center for Blood Research and Department of Pathology, Harvard Medical School, 800 Huntington Avenue, Boston, Massachusetts 02115, (USA), Fax + 1617 278 3368

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Abstract. von Willebrand disease is a genetic bleeding disorder that arises from abnormalities in von Willebrand factor, an adhesive glycoprotein involved in both primary hemostasis and coagulation. It is the most common inherited bleeding disorder in humans, and over the years several animal species have also been described as suffering from this disease whether through a spontaneous mutation (pigs, dogs) or a genetically engineered one (mouse). These different animal models are extremely useful in exploring the characteristics of von Willebrand disease and in testing new treatments. This review provides an update of the various von Willebrand disease models and the contribution that these models can make to a better understanding of human von Willebrand disease.

Key words. von Willebrand disease; von Willebrand factor; animal models; hemostasis; thrombosis; atherosclerosis.

Introduction

Human von Willebrand disease (vWd) was first described in 1926 by Erik von Willebrand [1]. The disease was characterized by frequent nose bleeds, prolonged bleeding times after minor surgery or from trivial wounds and excessive menorrhagia despite normal platelet counts and clotting time in vitro. This bleeding disorder could be distinguished from classic hemophilia A by its autosomal pattern of inheritance, the predominance of mucocutaneous bleeding and the prolonged bleeding time [2]. Further biochemical and immunologic characterization has shown that von Willebrand factor (vWf) and factor VIII (FVIII) (the protein whose absence leads to hemophilia A) are the products of two separate genes and that their qualitative and quantitative abnormalities cause two distinct bleeding disorders [3, 4]. The identification of the amino acid sequence of vWf [5] and the molecular cloning of its complementary DNA (cDNA) [6–9] have provided the basis for the elucidation of the molecular defects responsible for vWd. The human gene for vWf spans 178 kb and contains 52 exons [10], resulting in an 8.7-kb messenger RNA (mRNA). vWd can be classified in three major subtypes reviewed by Eweinstein [11]. Types 1 and 3 are caused by mild and severe quantitative defects in vWf, respectively, whereas type 2 is due to qualitative abnormalities.

vWf is a multimeric glycoprotein consisting of a series of dimeric subunits linked by disulfide bonds [12]. The molecular mass of vWf can range from 500,000 for the dimer to over 15×10^6 Da for the large multimers. vWf is synthesized by endothelial cells and megakaryocytes [13, 14]. Megakaryocyte-derived vWf is stored in the α-granules of platelets, whereas endothelial cell-derived vWf can be either released constitutively in the plasma or stored in specialized organelles called the Weibel-Palade bodies [15]. Upon activation of the endothelial cells by various agonists, the Weibel-Palade bodies will

* Corresponding author.

release mostly high molecular weight multimers of vWf, the forms that are the most active biologically [16].

vWf plays a critical role in primary hemostasis by binding to receptors on platelets and on exposed subendothelium forming a bridge at the site of vascular injury, leading to the formation of the platelet plug [17–19]. The role of vWf is particularly important at high shear rates, conditions encountered in arterioles and in microcirculation [20, 21]. Additionally, vWf mediates platelet-platelet interactions [22, 23]. In the coagulation process, vWf acts as a carrier for FVIII and protects it against inactivation by proteases [24].

Because of its important involvement in platelet adhesion and thrombus formation, vWf is likely to play a role in diseases in which these two physiological functions play a role, i.e. atherosclerosis, thrombotic thrombocytopenic purpura, disseminated intravascular coagulation, stroke, cancer metastasis, sickle cell disease and glomerular nephritis. Only with the help of vWd animal models can these questions be addressed in order to establish the precise role that vWf plays in these diseases [25].

Laboratory diagnosis of vWd

In order to validate a vWd model, a certain number of laboratory tests must be performed, and the results of these tests should be in accordance with the human disease. Some differences cannot be avoided, and it is important to adapt some of these tests to the model being studied.

Bleeding time

Bleeding time is the best indicator of primary hemostasis, and one of the most important features of vWd consists of a variably prolonged bleeding time [26–28]. However, this prolongation does not occur in midly affected individuals (type 1 vWd with a level of vWf antigen above 20%). This assay provides a good indication of the hemostatic status of individuals with vWd.

vWf antigen (vWf:Ag)

Enzyme-linked immunosorbent assay (ELISA) techniques are commonly performed today to measure levels of vWf in the plasma or in cell lysates [29]. Older techniques such as the Laurell rocket assay, radioimmunoassay (RIA) or immunoradiometric assay (IRMA) are not as popular now as they used to be. The vWf:Ag level is variably decreased in type 1 and almost undetectable in type 3 vWd. However, this assay does not really allow identification of type 2 vWd (the variants) which have a normal or closely normal vWf:Ag level but an abnormal function.

vWf activity

The ristocetin cofactor activity (vWf:RCoF) measures vWf function and is the most sensitive and specific assay for vWd [30]. Ristocetin is an antibiotic that promotes the interaction between vWf and its platelet receptor glycoprotein Ib. In this assay, the plasma to be tested is mixed in an aggregometer with ristocetin and a standardized source of normal or paraformaldehyde fixed platelets in order to induce platelet agglutination. In types 1 and 3 vWd, the vWf:RCoF usually parallels the level of vWf:Ag whereas in type 2 vWd there is a discordance, and vWf:RCoF is usually disproportionately reduced [31]. This test is not directly adaptable to all the different animal species. For example, vWf from porcine or bovine origin can aggregate human platelets in vitro without any agonist, suggesting some conformational differences between the human and porcine/bovine molecules [32, 33]. Comparison of the primary structure of the glycoprotein Ib binding domains of both human and porcine vWf revealed some differences that may account for the observed behavior [34]. A similar comparison was also done for bovine vWf [35].

Multimer analysis

The multimeric structure of vWf is extremely important since the high molecular weight multimers are shown to be the most active in their ability to support platelet adhesion [15]. Electrophoretic techniques allow visualization of the whole range of vWf multimers in a sample [12]. In type 1 vWd, there is an overall decrease in multimer levels but with a normal distribution of high, intermediate and low molecular weight forms. In type 3 vWd there is a lack of all types of multimers, and in type 2 vWd there is a wide variability of patterns, with certain forms lacking and other forms increased. This variability led to a more detailed classification of type 2 vWd [36]. High-resolution electrophoresis of human vWf shows a pentameric pattern with one central band and four satellite bands. This is also true for many other species except for dog, where each multimer is composed of one central band with only two satellite bands, suggesting some structural differences between human and canine vWf [37].

FVIII activity and coagulation assays

vWf acts as a carrier protein for FVIII in plasma and also plays a capital role in protecting it from degradation by proteases. Without this protection, FVIII is rapidly cleared from the circulation. Indeed, if FVIII alone is infused in patients with severe vWd or vWd dogs, its half-life is only 2.4 h, whereas it is about 12 h if vWf is infused at the same time [38]. In types 1 and 3 vWd, the level of FVIII closely parallels that of vWf

Table 1. Different animal species affected with vWd and characteristics of the disease.

Species	Breed/strain	vWd subtype	Mode of inheritance	vWf:Ag	FVIII (% of wild-type)	Genetic defect
Pig		Type 3	autosomal recessive	traces	30%	– reduced transcription – posttranscriptional defects
Dog	Numerous breeds	Type 1	autosomal dominant	variably decreased	variably decreased	defects in mRNA processing and stability
	German Shorthair Pointer	Type 2	undetermined	12–27%	reduced	unknown
	Scottish Terrier	Type 3	autosomal recessive	undetectable	15–50%	unknown
	Chesapeake Bay Retriever	Type 3	autosomal recessive	undetectable	18–30%	unknown
	German Wirehead Pointer	Type 3	autosomal recessive	traces	mildly reduced	unknown
	Dutch Kooiker	Type 3	autosomal recessive	traces	30–50%	G → A mutation in donor splice site of intron 16 ⇒ stop codon
	Shetland Sheepdog	Type 3	autosomal dominant	undetectable	73-77%	unknown
Mouse	RIIIS/J	Type 1	autosomal dominant	30–50%	30–50%	posttranslational modifications due to a mutation in a glycosyl-transferase ⇒ rapid clearance of vWf
	129Sv/C57Bl/6J or any breed	Type 3	genetically induced	0%	20%	gene targeting with insertion of the neomycin gene

despite a 50-fold excess of molecules of vWf in plasma compared with FVIII. In type 2 vWd, the level of FVIII is usually normal except in a particular variant (type 2N) which presents a defective binding of vWf to FVIII due to point mutations in the vWf gene.

As a result of the decrease of FVIII in vWd, the activated partial thromboplastin time may be abnormal. This assay detects abnormalities in the amplification of the coagulation cascade, and if the FVIII level is substantially decreased, the clotting time measured by that test is prolonged. In contrast, assays such as the prothrombin time, which detects abnormalities in the extrinsic coagulation pathway, is not affected.

Porcine vWd

A bleeding disorder was described in swine in 1941 by Hogan and colleagues [39]. Further studies demonstrated the similarities with human vWd such as the prolonged bleeding time and a low FVIII concentration, making the vWd pigs the oldest known animal model of a human bleeding diathesis [40–43]. The pig is a good model of hemorrhagic disorders since its clotting and platelet characteristics resemble those of humans. In normal pigs the level of vWf is close to the human level. Using human plasma as a reference (100%), the vWf:Ag level in pigs is about 100%, whereas in other animals such as cow, sheep or goat the levels of vWf:Ag reach 600–1200% [44].

Clinical evaluation

Bleeding time, measured by ear incision, is prolonged to more than 10 min in affected pigs, compared with about 2 min in normal pigs [45]. The FVIII level is decreased to about 30% of wild-type levels (table 1). Interestingly, pigs have a much higher concentration of various coagulation factors (FV, FVIII, FIX, FXI and FXII) compared with humans. Indeed, the FVIII level in a normal pig is about 700% compared with human FVIII levels [45]. The platelet counts were normal in vWd pigs, and ADP-induced aggregation was not different from that of normal pig platelets. Assessments of vWf:Ag and vWf:RCoF revealed values at the limit of detectability for the affected pigs, reinforcing the diagnosis of type 3, severe vWd [43].

Genetic analysis

An extensive study showed that the disease was transmitted as an autosomal recessive trait [46]. The bleeder swine are homozygous for the defect, whereas the carriers are heterozygous. The latter are usually asymptomatic, which renders difficult the evaluation of their

status. Indeed, they don't have a bleeding tendency and their FVIII levels are usually normal. However, their vWf:Ag and vWf:RCoF are reduced to 30–40% of normal [43]. It is of interest to note that in pigs, as opposed to humans, the level of FVIII does not follow the vWf:Ag very closely. The homozygous pigs are not totally deficient in vWf. Low, but significant amounts of vWf:Ag can be detected both in platelets and in endothelial cells from the pulmonary artery and from the inferior vena cava [47]. No gross gene deletion or rearrangement was identified in the vWf gene of vWd pigs, but the defect was shown to be tightly linked to the vWf locus, most likely representing a point mutation or small insertion/deletion within the vWf gene [48], which is located on the porcine chromosome 5 [49]. Analysis of the mRNA revealed a decrease in vWf message levels in vWd pigs down to one-third of wild-type levels [50]. However, the level of vWf mRNA detected in the vWd pigs is still significant and does not correlate with the very low amount of vWf:Ag. This fact indicates that posttranscriptional defects may also be involved, such as defects in translation or instability of the transcripts [50].

Platelet adhesion in vWd pigs

Platelets from normal pigs and vWd pigs were compared, and no differences were found relative to platelet size or number of α-granules [51]. The only difference observed was the absence of tubular structures within the α-granules in the vWd platelets, suggesting that these granule-associated tubules of normal platelets may represent the vWf molecule itself [52]. In order to assess platelet function, the formation of the hemostatic plug after ear incision was monitored in normal and vWd pigs [53]. In the affected pigs, although there was formation of large platelet aggregates, these aggregates were not efficient in stopping the bleeding due to their localization far away from the arterial laceration. Furthermore, these aggregates were penetrated by channels through which bleeding could continue [53]. This in vivo study emphasized the importance of vWf not only in the interaction of platelets with the blood vessel and in the localization of the hemostatic plug to the damaged vessel but also in platelet-platelet interactions as demonstrated in humans [22, 23]. The pig model was also used to measure platelet adhesion to damaged coronary arteries [54]. A similar platelet adhesion was observed in both normal and vWd pigs, but the platelets appeared less activated in the affected pigs, keeping a round morphology and fewer pseudopodia. The shear rate in coronary arteries is low, which could explain the absence of defect in platelet adhesion in the vWf-deficient pigs. That study uncovered a new role for vWf in platelet activation at low shear rate. The role of vWf in mediating platelet-vessel wall interactions at various shear rates was investigated using in vitro and ex vivo techniques [55]. Platelet deposition on pig thoracic aortae is reduced in the absence of plasma vWf at high shear rate ($> 850\ s^{-1}$), independent of the perfusion method used. At low shear rate ($424\ s^{-1}$), a defect was observed in the absence of plasma vWf only when heparinized blood was perfused ex vivo over the deendothelialized aorta.

Bone marrow tranplantation

The respective roles of plasma vWf and platelet vWf were addressed for the first time using crossed bone marrow transplantation in the pig model [56]. A normal bone marrow was transplanted in a vWd animal, resulting in a chimera with vWf-positive platelets and vWf-negative endothelium. The plasmatic compartment was only minimally replenished by the vWf in platelets, suggesting that most plasma vWf is endothelial-derived. Ear bleeding time was not consistently shortened, but after suffering hemostatic challenges, the transplanted pig was able to control its bleeding. Platelet vWf seems to improve only partially the hemostatic mechanism in severe vWd. Also, platelet vWf does not contribute to normal FVIII activity and cannot support occlusive thrombosis in response to stenosis and vessel injury [57]. In another study, the vWd pigs transplanted with normal bone marrow were transfused with vWf concentrate, restoring both the plasma and platelet vWf compartments [58]. Both the hemostatic response and thrombus formation were evaluated. One pig (out of two) presented a partial reduction in bleeding time (from > 30 min to 13.5 min), and using an ex vivo thombosis model, it was shown that at a shear rate of $1600\ s^{-1}$, platelet adhesion and thrombus size were normalized in these pigs [58]. Using the opposite approach, a normal pig transplanted with bone marrow from a vWd pig, it was suggested that plasma and subendothelial vWf are the major determinants of bleeding time since the bleeding time remains normal when vWf is absent from the platelet compartment [57]. From this latter transplantation experiment it was determined that platelets do not take up much vWf from plasma either by active or by passive absorption [59].

Role of vWf in atherosclerosis

Thrombogenesis and atherogenesis may be intimately linked [60], and it was suggested that platelets, by adhering to a damaged endothelial surface and releasing growth factors, may play a role in atherosclerosis [61–63]. Consequently, experimental animals known to have an impairment of platelet function were investigated to see if they would be less prone to develop atherosclero-

sis. Numerous studies have been done using vWd pigs [64]. Early studies showed a striking difference in the atherosclerotic lesions in the aorta between normal pigs and vWd pigs, both in spontaneous atherosclerosis and in diet-induced atherosclerosis [65]. Spontaneously, 7 control pigs out of 11 presented multiple or single raised fatty atherosclerotic plaques and intimal thickening, whereas only 1 vWd pig out of 11 had a significant plaque. However, the aortas of the vWd pigs presented flat fatty lesions characterized by subendothelial deposition of fat without intimal thickening [66]. After 6 months of atherogenic diet, all control pigs developed raised fatty atherosclerotic plaques, and most developed raised fibrous atherosclerotic plaques with important intimal thickening. In contrast, only 3 vWd pigs out of 7 developed significant raised fatty atherosclerotic plaques, which were smaller than those in the control pigs. Additional studies confirmed this protection against atherosclerosis in vWd pigs [67]. However it was noted in all these studies that normal pigs have a tendency to have higher levels of diet-induced hypercholesterolemia than do vWd pigs, a finding that was not systematically explored and that might have been of great importance. Indeed in one study, the amount of coronary atherosclerosis was shown to be related to the degree of hypercholesterolemia that the pigs develop and not to the presence of vWf [68]. The controversy about the involvement of vWf in atherosclerosis was further reinforced by a report by Nichols et al. [69] showing that the presence of a particular polymorphism at the apolipoprotein B100 locus can significantly influence the development of diet-induced hypercholesterolemia and coronary and aortic atherosclerosis in the pig, independent of the vWd status. From this study, the authors conclude that this polymorphism could have affected the results of the previous atherogenesis experiments in vWd animals. These results are in agreement with autopsy findings in three patients with vWd [70]. Atherosclerosis lesions, but no occlusive thrombosis, were present in patients with type 3 vWd. The patients' repeated transfusions of blood products containing vWf could account for these observations.

However, considering the vWf role in platelet adhesion and activation, a mechanism linking vWf to atherogenesis may still exist. It was shown that both pseudopod formation and spreading of platelets adhering to injured arterial walls was impaired in vWd pigs [71]. In order to investigate the role of vWf in occlusive arterial thrombosis, normal and vWd pigs were fed a high cholesterol diet, and at the end of the diet period coronary and carotid arteries were subjected to a stenosis/injury protocol to produce occlusive thrombosis [72]. Coronary atherosclerosis was present in both groups of pigs, but occlusive thrombosis failed to develop in vWd pigs despite the presence of atherosclerosis, severe hypercholesterolemia and the additional stenosis and injury. vWf may be required to support progression of platelet-fibrin microthrombi to occlusive arterial thrombosis. Additionally, prevention of occlusive thrombosis was also obtained in normal pigs after treatment of the animals with a monoclonal antibody to vWf [73].

In arteries with altered shear stress, such as one caused by a clamp applied on the vessel, the neointimal proliferation that occurs contains large amounts of vWf. But vWf presence is not required for the neointimal formation since it can develop similarly in arteries of vWd pigs [74]. This high local concentration of vWf could contribute to plaque thrombogenicity.

Treatment of the hemostatic defect

The hemostatic effect of a transfusion of vWf in vWd pigs was monitored. When porcine cryoprecipitate was infused, the vWf:Ag and vWf:RCoF increased rapidly but fell back to baseline in 12 h [75]. There is a delayed and sustained rise in FVIII level exceeding the amount that was infused [76]. A temporary shortening of the bleeding time was observed only when huge quantities of cryoprecipitate were infused. This study was in agreement with several observations made in patients with vWd where it was noted that the shortening of the bleeding time was much more transient than the persistence of FVIII after the infusion of plasma or cryoprecipitate [77, 78]. The porcine model was also used to test the efficacy of a human recombinant preparation of vWf [79]. A partial correction of bleeding time was obtained in only one pig, out of three, infused with the recombinant vWf. Bleeding time correction seems to be very hard to obtain, whatever the source of infused vWf. Indeed, in a vWd pig transplanted with normal marrow, no change in bleeding time was observed after infusion of porcine plasma derivative concentrate even though the level of vWf:Ag was brought to normal in platelets and in plasma. Perhaps subendothelial vWf is necessary to achieve correction of the bleeding time [59].

Summary

The porcine model has been studied extensively since its identification as a type 3 vWd model and has proved very useful. The pig is a good model because it is close to humans in many aspects. The vWf localization in endothelial cells and platelets mimics that of humans. The pigs can develop spontaneous atherosclerosis, and the lesion composition resembles that of humans. Furthermore, the lipoprotein profile is also close to the profile seen in humans, making the pig a good model to study atherosclerosis. However, there are the obvious disadvantages due to the size and housing cost of the animals. Furthermore, the pig is not completely defi-

cient in vWf, and the colony is not syngeneic. This last point is particularly important for study of the role of vWf in diseases involving several gene products, such as atherosclerosis.

Canine vWd

The first incidence of vWd in dogs was reported in 1970 in a German Sheperd family [80]. The results of the coagulation, platelet function and hemostasis tests performed indicated the similarities with human vWd. Over the years, many other dog breeds have been identified as suffering from this disease, making vWd the most common inherited bleeding disorder in dogs. However, under the term 'canine vWd', there seems to be a very heterogeneous group of diseases with different subtypes and mode of inheritance [81].

Clinical evaluation

More than 50 breeds of dogs have been reported to be affected by vWd [37]. An important feature in dogs is the quasi absence of vWf in platelets even in nonaffected dogs. In human beings, $\sim 10-20\%$ of total circulating vWf is located in the α-granules [82]. In contrast, canine platelets contain only 2% of total circulating vWf [83]. The multimeric structure of canine vWf is also slightly different from humans: in humans, each multimeric band is surrounded by four satellite bands, whereas in dogs only two satellite bands are present, suggesting differences in proteolytic processing after secretion [37]. Similar to humans, affected dogs are prone to mucosal or cutaneous bleeding. The clinical symptoms are numerous and all related to a bleeding diathesis. Hemorrhagic complications were usually associated with surgical procedures [81]. The diagnosis of vWd has been most often based on subnormal concentrations of vWf:Ag, which in normal dogs is ~ 6 μg/ml, a concentration close to the human vWf concentration. Bleeding time is variably prolonged whether measured by the cuticle bleeding time technique or the buccal mucosa bleeding time technique [84, 85]. Levels of FVIII are reduced in all cases of canine vWd, though not to the same extent as the levels of vWf:Ag (table 1). The FVIII-stabilizing activity of vWf does not seem to be as important in dogs as in humans. While type 3 patients have very low FVIII activities in the range of about 10% or even lower, FVIII activities around 20–50% are found in type 3 vWf-deficient dogs [86], but healthy dogs have an FVIII concentration about three times the concentration found in humans. As a consequence, the activated partial thromboplastin time is usually normal. Different breeds of dogs seem to be affected by different subtypes of vWd, and counterparts of all major types of human vWd have been recognized in the dog [81].

Type 1 vWd. As in humans, type 1 vWd is the most common and affects a large number of breeds. There is a wide range in the severity of symptoms. The inheritance appears to be autosomal dominant with incomplete penetrance [87]. Heterozygotes have plasmatic vWf:Ag less than 50% and may or may not present clinical evidence of vWd [88]. Homozygote dogs usually die of vWd at birth or soon thereafter [87]. It was observed that the disease becomes progressively less severe with advancing age and repeated pregnancies in the females [89]. In Doberman pinschers, a reduced constitutive release of vWf from endothelial cells was reported to be the cause of the vWf deficiency [90]. Together with a decrease in vWf mRNA in affected dogs, this result suggests that the defect affects expression of the vWf gene, mRNA processing or mRNA stability, a finding similar to that in some patients with type 1 vWd [91].

Type 2 vWd. Type 2 vWd is very rare in dogs and has been reported only in German shorthair pointers. The analysis of the multimers revealed an absence of the high molecular weight forms [92]. The disease is severe with recurrent hemorrhagic episodes.

Type 3 vWd. In the breeds affected with severe vWd, such as the Scottish terriers, no vWf:Ag or vWf:RCoF can be detected [93, 94]. The severe bleeders are homozygous for the vWd trait [95, 96]. FVIII activity varied between 15 and 50% (table 1). The heterozygotes are asymptomatic and are considered carriers [97]. The same characteristics apply to Chesapeake Bay retrievers, German wirehaired pointers and Dutch Kooiker dogs [98–100]. However, another affected breed, Shetland sheepdogs, is a little bit different in the fact that heterozygotes can also present some clinical bleeding problems [101]. Point mutations were identified in Dutch Kooiker dogs. The mutation responsible for the type 3 vWd phenotype was found to be a G to A transition at the first position of the donor splice site sequence of intron 16, resulting in a stop codon at position 729 in the propolypeptide of vWf [102].

Acquired vWd

Prolonged bleeding time and low plasma vWf:Ag can be observed in dogs suffering from an endocrine disorder such as thyroid insufficiency. Indeed, more than 70% of hypothyroid dogs have a decreased concentration of vWf:Ag [103]. Acquired forms of vWd have also been described in human beings, primarily associated with thyroid insufficiency and lymphoproliferative disorders [104, 105]. Acquired human vWd can also arise as a result of the production of antibodies that inactivate vWf [106]. Whether this could also be a cause of canine acquired vWd remains to be determined.

Contribution of the vWd dogs in research

In contrast to the vWd pigs, vWd dogs have not been used extensively for research purposes. However, one study has used dogs to study the role of vWf in arterial thrombosis [107]. In contrast to wild-type and hemophilia A dogs, vWd dogs do not develop occlusive thrombi following a stenosis and injury model of induced arterial thrombosis, even after infusion of canine vWf in the plasma, suggesting that both plasma and subendothelial compartments of vWf are required to support thrombosis.

Treatment of the hemostatic defect

Canine vWd can be satisfactorily managed by infusion of fresh, fresh-frozen canine plasma or cryoprecipitate [88]. After infusion of fresh frozen plasma or cryoprecipitate, the half-lives of vWf:Ag were 18.5 and 22 h respectively, similar to values reported in human patients [108]. The efficacy of recombinant human vWf was also tested in the dog model of type 3 vWd and was found to increase vWf:Ag, vWf:RCoF and FVIII and to decrease bleeding intensity [109]. Desmopressin acetate is a synthetic vasopressin analog that is used in humans to stimulate a rapid release of intracellular stores of vWf. This drug is used mostly for treatment of type 1 vWd [110]. Increases in plasma vWf in response to desmopressin are less pronounced in clinically normal dogs than in human beings [111, 112]. Thus, desmopressin has not been proven effective in dogs affected with type 1 vWd [113].

Summary

Canine vWd is a group of bleeding diseases which can vary in genetic transmission, clinical severity and diagnostic laboratory findings. All purebred, as well as mixed breed, dogs have some apparent risk for vWd. Some breeds have a high prevalence of the disease (15–60% frequency), whereas some other breeds such as Airedale terriers have plasma vWf:Ag concentrations below the range for normal dogs without signs of abnormal bleeding [81]. This breed-to-breed variability makes it difficult to identify the asymptomatic carrier or heterozygous state based solely on vWf:Ag concentration. This issue is particularly important for breeders in order to avoid mating two carrier parents and producing severely affected progeny. Differences in clinical manifestations of vWd in purebred dogs may reflect heterogeneous defects within the vWf gene, causing a variety of abnormalities in production, structure and function of vWf protein. Analogous to human vWd, acquired deficiencies of vWf may also contribute to the clinical variability of vWd in dogs.

Murine vWd

Two murine models of vWd have been described. One is a naturally occurring model of vWd, the RIIIS/J mouse [114], whereas the other one is a genetically modified model obtained by gene targeting and disruption of the vWf gene [115].

The RIIIS/J mouse

Clinical description. The defect in this mouse strain was discovered during random testing of bleeding times in 25 common strains. A prolonged bleeding time (>15 min) was a hallmark of the RIIIS/J mouse strain, and more extensive studies of these mice were undertaken. Plasma vWf:Ag was reduced to one-half to one-third of the level found in normal mouse plasma. FVIII was similarly reduced, and the activated partial thromboplastin time was prolonged 2.5-fold (table 1) [114]. However, platelet vWf concentration was similar to that of normal mice, and no storage pool deficiency was found. Platelet aggregation was normal in the presence of ATP, collagen or ristocetin. The RIIIS/J mice responded to desmopressin by a reduction in their bleeding time, but surprisingly this effect was sex-dependent and observed only in females. The multimers were all present although reduced in concentration, a sign of type 1 vWd. No significant spontaneous hemorrhage was observed among the mouse colony.

Identification of the genetic defect. The inheritance of the defect appears to be autosomal dominant. Interestingly, when the RIIIS/J mice were bred to another strain, the progeny had vWf:Ag concentrations identical to the affected RIIIS/J parents instead of values between those of the two parents. This result was the first indication that the genetic defect in RIIIS/J mice may involve a regulatory gene rather than the structural gene for vWf [114]. A second report described genetic linkage analysis of vWd in the RIIIS/J mice, confirming that murine vWd in these mice is caused by a defect at a novel genetic locus distinct from the murine vWf gene [116]. A recent study identified that defect to be caused by a switch in cell-type-specific expression of the *Galgt2* gene from gastrointestinal cells to vascular endothelial cells in RIIIS/J mice [117]. *Galgt2* gene encodes a glycosyltransferase enzyme, GALGT2, and the switch in its pattern of expression leads to GALGT2-mediated transfer of *N*-acetylgalactosamine onto the glycans that decorate endothelial cells-synthesized proteins, including vWf. The result of this aberrant posttranslational modification is a rapid clearance of vWf from the circulation (fig. 1). The conclusion drawn from that study carries important implications for human vWd, where it was suggested that approximately 60% of the variation in human plasma vWf:Ag level is determined by genetic

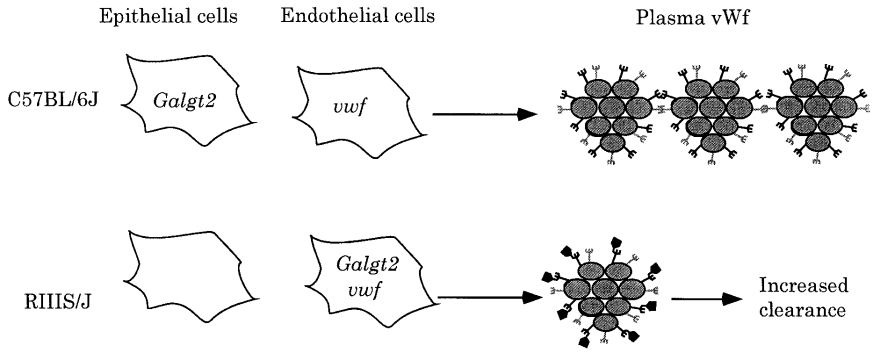

Figure 1. Model for mechanism of the modifier gene action in RIIIS/J mice. Galgt2 is normally expressed in intestinal epithelial cells and vwf in endothelial cells. In RIIIS/J mice, the expression of Galgt2 is switched to endothelial cells. This switch leads to the transfer of *N*-acetylgalactosamine (black pentagons) onto oligosaccharides on vWf. This novel sugar structure leads to increased clearance of vWf, resulting in decreased plasma levels. Reprinted with permission from K. L. Mohlke, A. A. Purkayastha, R. J. Westrick, P. L. Smith, B. Petryniak, J. B. Lowe and D. Ginsburg, Cell, 1999, **96:** 111–120.

factors. One of the important factors is the effect of the ABO blood type, people with the O blood type having lower levels of vWf [118]. It is very tempting and plausible to imagine that, similar to R III S/J mice, alterations in posttranslational processing, including glycosylation, could represent an important mechanism for genetic modifiers of human vWd and perhaps other diseases involving secreted proteins.

The vWf knockout mouse

Using gene targeting techniques, a mouse model for type 3 vWd was engineered. Insertion of the neomycin resistance gene in the murine vWf locus disrupted the gene, and no trace of vWf protein is detectable in the plasma, platelets and endothelium of these mice [115]. Their bleeding time is prolonged and often infinite (table 2). The activated partial thromboplastin time is prolonged in the vWf-deficient mice. Their FVIII level is reduced to about 20% of that found in wild-type mice (table 1). Interestingly, the vWf heterozygous mice, which have a level of vWf:Ag of 50%, also have a reduced FVIII level, between 50 and 60% of wild-type. This situation mimics human type 1 vWd and makes this mouse a good model to study the regulation of FVIII by vWf. The mice in the vWf-deficient colony do not suffer major bleeding problems, but there are some indications of spontaneous bleeding occurring particularly in pups. About 10% of the vWf-deficient neonates develop intraabdominal bleeding that could potentially lead to death.

Local Shwartzman reaction

In order to test the hemostatic capacities and the inflammatory response of vWf-deficient mice, we studied a model of hemorrhagic vasculitis which shows the close interrelation between the inflammatory and hemostatic systems. The local Shwartzman reaction, modified from the original method described by Shwartzman [119], was induced by injection of lipo-poly-saccharide (LPS) followed by injection of TNF-α in the same skin site day later [120]. Twenty-four hours after the last injection, the lesions were evaluated macroscopically [121]. The size of the lesions in vWf-deficient mice was two times larger than in the wild-type mice. The increased hemorrhage seen in

Table 2. Hematological and coagulation analysis of vWf-deficient mice

	+/+	+/−	−/−
vWf Antigen (%)	96 ± 5.6	49 ± 3.6*	not detectable
FVIII activity (%)	139.5 ± 7.5	81 ± 4*	27.6 ± 1.1*
PT (s)	14.7 ± 0.42	14.8 ± 0.63	14.9 ± 0.67
aPTT (s)	23.2 ± 0.5	24.4 ± 0.4	34.5 ± 1*
Bleeding time (s)	69.7 ± 5.2	91.9 ± 11.2	499 ± 33.4*
Platelets ($\times 10^9$/l)	837 ± 43	986 ± 86	931 ± 27
RBC ($\times 10^{12}$/l)	7.88 ± 0.12	7.67 ± 0.21	7.72 ± 0.17
WBC ($\times 10^9$/l)	2.8 ± 0.33	3.37 ± 0.36	3.07 ± 0.34
Hematocrit (%)	37.1 ± 0.6	35.4 ± 1.1	36.9 ± 1
Hemoglobin (g/dl)	12.8 ± 0.24	12.4 ± 0.22	12.7 ± 0.31

$n = 8$–13 mice except for the bleeding time where $n = 15$–21. All mice were 2–3 months of age. For the ELISA and FVIII activity data, 100% was defined as being the percentage of antigen or activity found in a pool of plasma from 10 wild-type mice. * $p < 0.0001$ when compared with wild type. Reprinted with permission from C. Denis, N. Methia, P. S. Frenette, H. Rayburn, M. Ullman-Culleré, R. O. Hynes and D. D. Wagner, Proc. Natl. Acad. Sci. USA, 1998, **95:** 9524–9529.

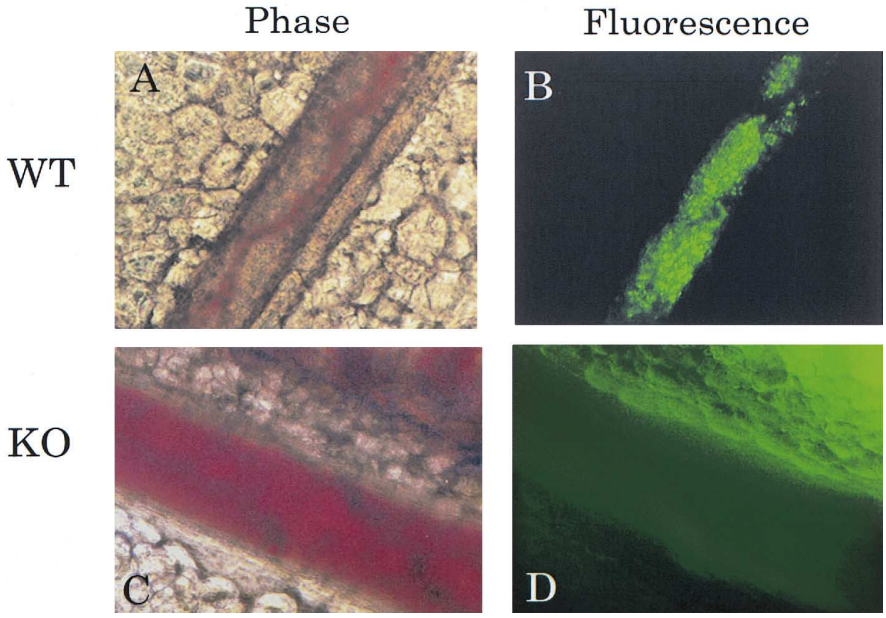

Figure 2. In vivo thrombosis model in arterioles after ferric chloride injury. An arteriole from a wild-type (WT) (*A*, *B*) or a vWf-deficient mouse (KO) (*C*, *D*) was injured by ferric chloride superfusion and photographed, using phase and fluorescent filters, 10 min after injury. The wild-type vessel is almost completely occluded by platelet thrombi, whereas almost no platelet interactions are visible on the vWf-deficient arteriole.

vWf-deficient mice was confirmed by microscopic evaluation of skin sections from the site of the reaction. In the wild-type animals there were fewer erythrocytes in the tissues than in the mutant mice, in which significantly more erythrocytes were seen throughout the tissues surrounding the blood vessels [C. Denis and D. D. Wagner, unpublished observations]. The endothelial damage caused by the cytokines and other factors results in leakage of red blood cells [122]. vWf present in the subendothelium may contribute to the adhesion of endothelial cells, helping to maintain endothelial integrity [123]. Therefore, a lack of subendothelial vWf may result in an increased vascular fragility and permeability, an issue worthy of further investigation with these animals. Also, once the damage is done, the platelets will not repair the injury as efficiently, due to their decreased interaction with the vessel wall in the absence of vWf [115].

Thrombosis was also evaluated by counting the percentage of vessels partially or completely occluded by thrombi composed of fibrin, platelets and neutrophils. There was a significant decrease in the number of visibly occluded vessels in the vWf-deficient sections as compared with wild-type [C. Denis and D. D. Wagner, unpublished observations].

In vivo thrombosis model. Thrombosis formation in the vWf-deficient mice was monitored using intravital microscopy [115]. Mice were injected intravenously with fluorescently labeled platelets of the same genotype as the recipient, and the mesenteric arterioles (60–100 μm) were exteriorized and filmed. Arterioles were chosen for their high shear rate, conditions in which the vWf-glycoprotein Ib axis is essential for the first steps of platelet thrombus formation. Endothelium injury was provoked by superfusion of ferric chloride [124] on the arteriole, and the injured vessel was monitored. In the wild-type animals, platelet interactions with the injured vessel wall started very quickly, and after 10 min all of the wild-type arterioles presented either complete occlusion (25%) or numerous platelet interactions with the vessel wall, including formation of thrombi. An example of a wild-type arteriole is shown in figure 2A and 2B where the vessel lumen is almost totally occluded with thrombi, leaving only a small channel for the blood to pass and which too will soon occlude. In contrast, in the mutant animals most arterioles (66.6%) had very few, if any, platelet interactions with the vessel wall during the first 10 min of recording [115]. A typical example of a vWf-deficient arteriole is shown in figure 2C and 2D. The average occlusion time for wild-type vessels in this model is 14.7 ± 2 min ($n = 12$). Forty minutes after injury, only 50% of vWf-deficient arterioles reached complete occlusion. In the remaining vWf-deficient vessels, the platelet thrombus

stopped growing, leaving an open channel allowing blood flow to continue [H. Ni and D. D. Wagner, unpublished observations).

Summary

The mouse model has many advantages compared with the pig and dog models. The small size of the animals makes it a relatively inexpensive model, allowing large numbers of animals to be evaluated and meaningful statistical analyses to be performed. Because the mutation can be placed onto inbred strains, the role of vWf in complex diseases such as cancer or atherosclerosis can be specifically addressed. All these reasons make the mouse the current of model choice for mammalian studies.

Conclusion

In this review we have described the vWd models most commonly studied and reported in the literature; but vWd has been described in other animal species such as rabbits [125, 126] and cats [127]. It seems that the size of the vWf gene makes it a particularly large target for the occurrence of random mutations, possibly contributing to the frequent occurrence of vWd in humans and identification of a similar disease in a number of animal species. These different vWd models, both inherited and induced, continue to advance the knowledge of vWf-related diseases. There are, however, some differences between the various models, and one should be cautious about extrapolating the results from animals to humans. Perhaps there can be no one standard model, but rather each model must be selected to fit the type of pathophysiologic thrombotic mechanism being studied. The differences between models can sometimes be used to our advantage. For example, since dogs appear to express vWf in their plasma but not in their platelets, it is possible that comparison of regulatory regions of human and pig vWf genes to the canine vWf gene could elucidate the factors responsible for platelet expression of vWf [37]. The role of the different compartments of vWf in thrombosis and hemostasis can also be addressed using the vWd models. For dogs it seems that plasma and subendothelial vWf are the key players for the development of thrombosis, whereas in pigs, plasma and platelet vWf play the major roles. These differences can explain why bleeding time shortening following vWf infusion differs among vWf-deficient species. Indeed, in canine vWd, infusion of FVIII-deficient cryoprecipitate corrects the bleeding time [107], whereas in porcine and human severe vWd, infusion of both plasma and platelet vWf is required to normalize the bleeding time [58, 128]. Crossed bone marrow transplantation using the vWf-deficient mice and wild-type mice will provide information on the individual contributions of platelet and endothelial vWf in this species. Use of vWd animal models will also allow us to address the question of FVIII regulation by vWf. In human type 1 vWd, the concentration of FVIII usually parallels that of vWf even though there is a considerable molar excess of binding sites provided by the remaining vWf [129, 130]. The close relationship between the plasma concentrations of the two proteins is still a mystery. In patients who are heterozygous for FVIII binding defect (vWd type 2 Normandy) [131] and who have only half of the normal binding sites for FVIII but normal total vWf antigen levels, the FVIII levels appear normal [132]. This indicates that FVIII binding to vWf is not the complete story. Perhaps a feedback mechanism exists between the vWf plasma level and the biosynthesis/secretion of FVIII. An animal model should be very useful for assessing this question. A recent study investigated this issue using vWd pigs [133]. Infusion of vWf into a vWf-deficient pig produces a fivefold increase of circulating FVIII activity, but no difference in the level of FVIII mRNA in the liver is observed. However, FVIII mRNA is also detected in many tissues besides the liver [134], and differences in mRNA levels could be found elsewhere. Furthermore, it may be important to infuse vWf propolypeptide along with mature vWf. The vWf prosequence levels in blood might more accurately reflect the activation state of the vasculature [135], so regulating FVIII levels by the prosequence would make biological sense.

The FVIII activity in the plasma of animals appears higher than that of human plasma: seven times higher in pigs [45], two to three times in dogs [113] and in mice [114, 115]. In the large animal models of severe vWd, it was observed that the levels of FVIII were highly variable and not as much decreased as in humans with severe vWd where they drop to less than 10% of normal value [11]. FVIII levels in vWd dogs are usually above 50% of those in wild-type dogs [109], and in the vWd pigs the level of FVIII varies between 12 and 60% of that of wild type [45]. It appears that in these animals FVIII does not require vWf for protection as critically as it does in humans, due to either lower susceptibility to proteolysis or to the presence of other molecules that can provide protection. In contrast, the vWf-deficient mice are a good model to study the regulation of FVIII by vWf. Their FVIII level is relatively low (20% of wild type) and there is very little variation between animals (table 2). In addition, the heterozygous mice, which have half-normal amounts of vWf antigen, also have a decreased FVIII activity down to 57% of the wild-type. These vWf-deficient mice will help us to understand the close relationship between vWf and FVIII and also to clarify whether there is a storage compartment for

FVIII, since FVIII is released into the blood simultaneously with vWf after infusion of the vasopressin analogue DDAVP used in patients to rapidly elevate vWf levels [110].

Besides decreased FVIII levels, another secondary defect associated with severe vWd may involve P-selectin, the other protein localized in Weibel-Palade bodies. The tubular structure of these granules seems to be due to a high, perhaps crystalline, organization of the vWf protein [136]. In the absence of vWf, it is not clear whether functional Weibel-Palade bodies can be formed. P-selectin is an important mediator of leukocyte recruitment at sites of inflammation [137], and its mislocalization could lead to changes in leukocyte extravasation. Animals models such as the mouse lacking vWf should be useful to test this hypothesis.

The mouse model has the huge advantage that these animals can be bred to other mice strains in order to create additional models. Numerous adhesion molecule-deficient mice or coagulation protein-deficient mice have been generated over the past decade. By breeding some of these mice together, the scientific community now have a wide variety of models at their disposal. Breeding vWf-deficient mice with fibrinogen-deficient mice will lead to the ablation of the two proteins thought to be involved in thrombus formation at high and low shear rate. Such studies are now in progress. In order to clarify the controversy about the possible involvement of vWf in atherosclerosis, vWf-deficient mice can be bred to mice strains susceptible to this disease such as Apo-E-deficient mice or Low Density Lipoprotein (LDL) receptor-deficient mice.

In conclusion, there are several different models of vWd currently available and which have already proven very useful in characterizing this disease. Many questions still remain unanswered, such as the involvement of vWf in pathological conditions involving platelet adhesion and thrombus formation, but no doubt the recently generated mouse model will allow them to be addressed. Development of better treatments for vWd is the ultimate goal, and these animal models can be used to test the potency of various vWf/FVIII preparations, agents stimulating vWf synthesis or secretion and eventually gene therapy methods.

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