

Function of von Willebrand factor after crossed bone marrow transplantation between normal and von Willebrand disease pigs: Effect on arterial thrombosis in chimeras

(animal model/chimerism/factor VIII/platelets)

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ABSTRACT von Willebrand factor (vWF) is essential for the induction of occlusive thrombosis in stenosed and injured pig arteries and for normal hemostasis. To separate the relative contribution of plasma and platelet vWF to arterial thrombosis, we produced chimeric normal and von Willebrand disease pigs by crossed bone marrow transplantation; von Willebrand disease (vWD) pigs were engrafted with normal pig bone marrow and normal pigs were engrafted with vWD bone marrow. Thrombosis developed in the chimeric normal pigs that showed normal levels of plasma vWF and an absence of platelet vWF; but no thrombosis occurred in the chimeric vWD pigs that demonstrated normal platelet vWF and an absence of plasma vWF. The ear bleeding times of the chimeric pigs were partially corrected by endogenous plasma vWF but not by platelet vWF. Our animal model demonstrated that vWF in the plasma compartment is essential for the development of arterial thrombosis and that it also contributes to the maintenance of bleeding time and hemostasis.

von Willebrand factor (vWF) is a large adhesive glycoprotein that is synthesized in the endothelial cells (1, 2) and megakaryocytes (3, 4) and present in platelet α granules (3, 5–7). Plasma vWF carries factor VIII (F.VIII) (8), prolongs its half-life (9–11), protects it from degradation by activated protein C (12, 13), functions as a cofactor in thrombin-dependent cleavage of the F.VIII light chain (14), and modulates the activation of F.VIII and factor X (15, 16). The absence of vWF produces von Willebrand disease (vWD), an inherited and sometimes fatal bleeding disorder. vWF supports platelet adhesion to vascular subendothelium at high shear rates (17) and supports spreading of adherent platelets *in vivo* in pigs (18, 19) and in *ex vivo* models of platelet adhesion (20–22). In addition, vWF supports the development of occlusive arterial thrombosis in stenosed and injured arteries in animal models (23–26). What remains incompletely understood is how the vWF in the individual plasma and platelet compartments supports these diverse processes in thrombosis and hemostasis.

The role of plasma and platelet vWF in hemostasis relates in part to the compartmentalization or cellular source of vWF, and it also varies among species. Studies in humans and pigs demonstrate that bleeding time prolongation is associated with decreased platelet vWF, independent of changes in plasma vWF (5, 27–30). Interestingly, for porcine vWD and human

type III vWD, infusion of plasma and platelet vWF is required to normalize the bleeding time, whereas infusion of plasma vWF alone is insufficient (5, 31–34). In contrast, infusion of F.VIII-deficient canine cryoprecipitate largely corrects the bleeding time in canine vWD (26). Moreover, normal dogs lacking platelet vWF but possessing normal plasma and endothelial vWF exhibit normal bleeding times (26). Bleeding time correction thus requires infusion of plasma and platelet vWF in pigs and humans with vWD; but in normal and vWD dogs, plasma vWF alone is sufficient.

Normal and vWD pigs make excellent models for the study of the relative contribution of plasma and platelet vWF to arterial thrombosis, especially since vWF has proven critical for induction of thrombosis in pigs (23–25). Pigs with vWD are the oldest known animal model of a human bleeding diathesis (35–38). These pigs have an autosomally inherited bleeding disease (39) that is similar to human type III vWD (40, 41). We chose bone marrow transplantation as a method for producing chimeric animals whose vWF is restricted to either plasma or platelets. Transplantation of the wild-type porcine vWF or vWD gene in previous studies altered the expression of vWF in the plasma and platelets of recipient pigs (5, 29, 42). The purpose of producing these animals was to determine the individual contribution of vWF in the plasma or platelet compartments to thrombosis and hemostasis (43). In this paper, the plasma vWF compartment in the transplanted pigs also includes the endothelial and subendothelial compartments (2, 44, 45). The results show that vWF can be isolated in these two compartments by bone marrow transplantation between normal and vWD pigs, that plasma vWF in the absence of platelet vWF supports thrombosis and partially corrects bleeding time, and that platelet vWF by itself cannot support thrombosis or bleeding time.

MATERIALS AND METHODS

Experimental Animals. Normal ($n = 10$) and vWD ($n = 10$) pigs came from the colony at the Institut National de la Recherche Agronomique, Jouy-en-Josas, France (Fig. 1). This colony of vWD pigs was established by a gift of E. J. W. Bowie, (Rochester, MN). Eight normal and eight vWD pigs were used for donor and recipient pigs in bone marrow transplantation

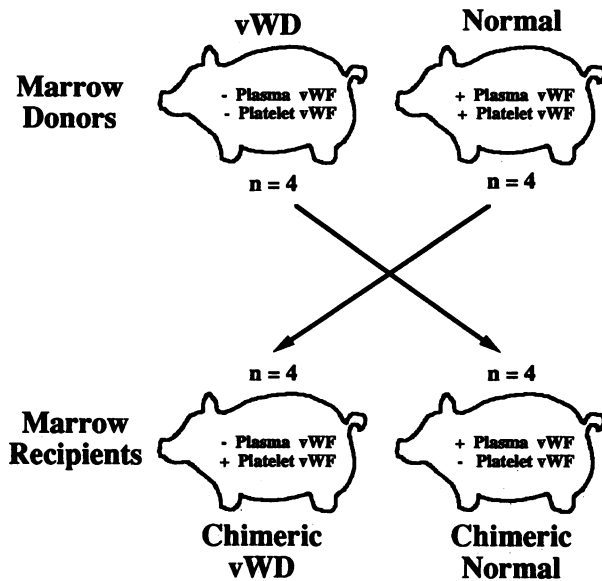


FIG. 1. Crossed bone marrow transplantation between normal and vWD disease pigs to produce chimeras. Twelve pigs were used in our study (six vWD and six normal). Eight chimeric pigs were produced. Four pigs (two vWD and 2 normal) were used as nontransplanted controls.

studies (Fig. 1). Two normal and two vWD pigs served as nontransplanted controls in our studies.

Plasma and Platelet vWF and F.VIII Assays. Platelet-poor plasma, platelet rich plasma, washed platelets, and platelet lysates were prepared and analyzed for vWF activity as described (19, 26, 39). vWF-antigen (vWF·Ag) multimers were separated and analyzed (26, 46). vWF·Ag was assayed by ELISA (2, 7). F.VIII coagulant activity was performed by a modified one-stage method (26, 47). One unit of either vWF or F.VIII activity equaled the amount present in 1 ml of normal pig pooled plasma.

Bleeding Time. Saline ear bleeding times were performed on all pigs (36).

Bone Marrow Transplantation in Pigs. Two phenotypic variants of pigs were produced using a strategy of cross bone marrow transplantation (Fig. 1): (i) *Chimeric vWD pigs with platelet vWF.* Briefly, vWD pigs ($n = 4$) were irradiated to kill native bone marrow cells and then transplanted with bone marrow from a swine leukocyte Ag-matched normal pig (48–50). Bone marrow recovery in these pigs will be described elsewhere (unpublished data by one of the coauthors, J.R.). No pig received immunosuppressive therapy, and the transplanted pigs were subjected to thrombosis studies described below within 2 months of transplantation. (ii) *Chimeric normal pigs with no platelet vWF.* Normal pigs ($n = 4$) were irradiated and then transplanted with bone marrow from a vWD pig matched immunologically in an identical fashion to the chimeric vWD pigs.

Chromosome Analysis. All transplanted pigs received marrow from a pig of the opposite sex. Successful engraftment of all chimeras was documented by identifying the karyotype of the donor's sex in cultured bone marrow cells (51).

Arterial Stenosis and Injury Procedure: Animal Preparation, Histological Studies, and Vessel Morphometry. The carotid and coronary arterial stenosis and injury procedure was performed using a modified procedure of Folts and Uchida (23–26, 52–54). Arteries were pressure-perfused and placed in fixatives. The degree of arterial stenosis and injury was measured by computer-assisted planimetry, and the presence or absence of thrombosis was determined by examining arterial sections with light microscopy (23–26). Coronary and carotid artery results were grouped for analysis since we

previously found equivalent incidences of thrombosis in the two circulations relative to the presence or absence of vWF (23, 24), and in this study, all pigs demonstrated the same outcome for their carotid and coronary arteries.

Infusion of vWF. Porcine vWF devoid of porcine F.VIII (Porton Speywood, Ltd., Birmingham, England) was infused over 30 min. F.VIII activity, vWF activity, Ag, and multimeric distribution were determined for each infusate and for plasma samples collected every 30 min during the stenosis and injury procedure.

Evaluation of Release of vWF by Adherent Platelets in Baumgartner Annular Perfusion Systems. Platelets circulating in the chimeric vWD pigs were evaluated for their ability to adhere to exposed subendothelium and release of vWF at a shear rate of 1700 sec^{-1} (24). The Baumgartner system was constructed to contain (i) an artery from a vWD pig and whole blood from a chimeric vWD pig expressing normal platelet vWF, (ii) an artery from a vWD pig and whole blood from a wild-type vWD pig expressing neither platelet nor plasma vWF, and (iii) a normal porcine artery and normal pig blood. The perfused arterial segments were evaluated for vWF deposition from platelets by an immunogold labeling procedure (55).

Statistical Methods of Analysis. All measured variables are reported individually and as mean values for groups. The Wilcoxon rank sum test was used to compare the two transplanted pig groups for variables with ordinal or interval levels of measurement. Unless otherwise noted, P values are from these tests. Fisher's exact test was used for comparisons concerning dichotomous variables.

RESULTS

Characterization of Chimeric Normal Pigs. The strategy of transplanting vWD bone marrow into normal pigs was utilized to produce chimeras that expressed normal plasma vWF but no platelet vWF (Fig. 1). Plasma from these chimeric normal pigs had normal vWF Ag and activity and a full range of multimers (Table 1; Fig. 2). The platelet lysates from these pigs had no vWF activity or Ag and no vWF multimers (Table 1; Fig. 2). The function of isolated plasma vWF in the absence of platelet vWF was then tested in these chimeric normal pigs. First, arterial thrombosis in response to stenosis and injury developed in six of six arteries (Table 1, three of three coronary, three of three carotid). By comparison, four of four arteries (two of two coronary, two of two carotid) tested in two normal control pigs developed thrombosis with stenosis and injury. Second, the saline bleeding time (SBT) exhibited a range from

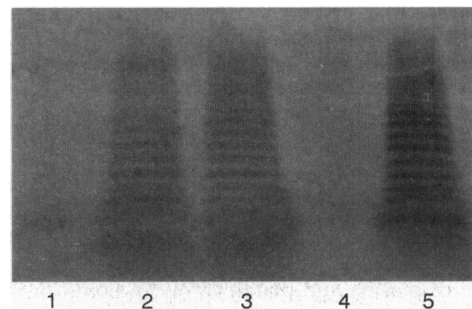


FIG. 2. Multimeric distribution of vWF·Ag in the plasma and platelets in chimeric normal and vWD pigs following bone marrow transplantation. Lanes 1 and 2, platelet lysates and plasma samples, respectively, from pig no. 95080, a normal pig grafted with vWD pig bone marrow. Lanes 3 and 4, platelet lysate and plasma samples, respectively, from pig no. 95136, a vWD pig grafted with normal pig marrow. Lane 5, normal pig pooled plasma for comparison. The range of multimers in the compartments with vWF is equivalent to that seen in normal pig pool plasma.

Table 1. Characterization of normal pigs: Chimeric and wild type

Parameter	Normal	
	Chimeric (n = 4)	Wild type (n = 2)
Platelet vWF*		
Ag	<1	ND†
Activity	<1	145
Multimers	Absent	Full range
Plasma vWF*		
Ag	163.8 ± 120.2	180
Activity	91.3 ± 9.8	165
Multimers	Full range	Full range
Thrombosis‡	6/6	4/4
Bleeding time (min)	6.1 ± 2.2	3.8 ± 1.5
F.VIII*	97.3 ± 2.5	123
Platelet count (per μl)	391,000 ± 63,100	438,000

*vWF activity, vWF Ag, and F.VIII are expressed as a % of normal pig plasma pool. The values listed for the transplant recipients were determined using samples taken at the time of the terminal experiment, which was at least 2 weeks after receiving any blood products. The values listed for the two vWD control pigs are the average of the two individual values. Distributions of hematocrits were similar among groups in Tables 1 and 2 (data not shown).

†ND, not done. The average platelet vWF:Ag in normal pigs at Jouy-en-Josas is 238 ± 113 (n = 10).

‡These values indicate the number of stenosed and injured arteries that developed thrombosis per total number of arteries tested. The degree of stenoses induced by the Goldblatt clamp and injury varied over a large range in all phenotypes, for wild-type normal and vWD pigs and for both groups of chimeric pigs (data not shown).

3.5 min to 8.5 min in these chimeric normal pigs (mean, 6.8 ± 2.2, Table 1). The average SBT in wild-type normal pigs is 3.8 ± 1.5 min. Finally, F.VIII activity was unchanged in the absence of platelet vWF (Table 1). Thus, plasma vWF by itself supports thrombosis, helps correct bleeding time, and contributes to normal F.VIII activity.

Characterization of Chimeric vWD Pigs. We grafted normal bone marrow into vWD pigs to produce chimeric vWD pigs that had normal bone marrow and thus expressed platelet vWF but no plasma vWF (Fig. 1). Platelet lysates from chimeric vWD animals expressing normal platelet vWF had mean vWF Ag and activity values of 211% and 110%, respectively (Table 2). Also, a full range of vWF multimers was detected in platelet lysates (Fig. 2). Plasma from the chimeric vWD pigs grafted with normal bone marrow had <1% vWF Ag and <1% vWF activity following transplantation (Table 2). There were no detectable plasma vWF:Ag multimers in this group following transplantation (Fig. 2). The function of isolated platelet vWF in the absence of plasma vWF was then tested in these chimeric normal pigs. First, arterial thrombosis in response to stenosis and injury developed in none of the seven arteries of the

Table 2. Characterization of vWD pigs: Chimeric and wild type

Parameter	vWD	
	Chimeric (n = 4)	Wild type (n = 4)
Platelet vWF		
Ag	211 ± 133.6	<1
Activity	110 ± 56.1	<1
Multimers	Full range	Absent
Plasma vWF		
Ag	<1	<1
Activity	<1	<1
Multimers	Absent	Absent
Thrombosis	0/7	0/4
Bleeding time (min)	>15	>15
F.VIII	7.2 ± 5.9	8
Platelet count (per μl)	391,000 ± 63,100	498,000

Values are listed in an identical fashion to those in Table 1.

chimeric vWD pigs (Table 2, zero of three coronary arteries, zero of four carotid arteries). The difference between the incidence of thrombosis between chimeric vWD and chimeric normal pigs is significant (one-sided *P* = 0.029 for percentage of tested arteries with thrombosis per pig, Fisher's exact test; and two-sided *P* < 0.01 for tested arteries in their own right, Fisher's exact test). Second, the SBT was >15 min (Table 2). Finally, F.VIII activity was depressed to a level equal to that found in vWD control pigs (Table 2). Thus, platelet vWF by itself does not support thrombosis, correct bleeding time, or contribute to normal F.VIII activity.

Platelet Function in Chimeras. The function of platelets produced by transplanted megakaryocytes in the chimeric pigs was investigated in a Baumgartner annular perfusion chamber. Platelets from chimeric vWD pigs with normal platelet vWF were shown to adhere to vascular subendothelium in an annular perfusion chamber by transmission electron microscopy (TEM) (Fig. 3A). These platelets revealed immunogold deposition in granules and on their surface in a pattern that was identical to normal platelets adherent to normal pig arterial subendothelium (Fig. 3B). By comparison, adherent platelets from a chimeric normal pig transplanted with vWD marrow revealed no immunogold deposition in granules or on the membrane surface (Fig. 3C). Thus, platelets produced by transplanted megakaryocytes in the vWD chimeras were capable of attaching to vascular subendothelium and releasing vWF.

Effect of Infusion of F.VIII-Free Porcine vWF into Chimeric vWD Pigs on Hemostasis and Thrombogenesis. Porcine vWF was infused into chimeric vWD pigs expressing platelet vWF to investigate the possibility of an interaction between the vWF in the two compartments on bleeding time and thrombosis (Table 3). In these three chimeric vWD pigs, plasma vWF activity and Ag increased and a full range of multimers of vWF:Ag was detected in the infusate and in the plasma following infusion (Table 3). The distribution of high molecular weight multimers was comparable by laser densitometry to that present in normal pig pool plasma (data not shown). Despite the presence of vWF functionally and antigenically in the plasma and platelets, the SBT remained >15 min in all three pigs and none of the six arteries developed evidence of thrombosis following stenosis and injury (Table 3). There was <1% F.VIII activity in all three samples of the porcine vWF infusion product. Mean F.VIII levels in the pigs rose only slightly in the infused animals, from 7.2% to 22% with monitoring for up to 2 hr. The infused porcine vWF product did not correct bleeding time or support thrombosis in these chimeras.

DISCUSSION

The purpose of this study was to produce pigs with vWF isolated to the plasma or platelet compartments and to determine the contribution of vWF in these individual compartments to thrombosis and hemostasis. The strategy of crossed bone marrow transplantation between normal and vWD pigs was chosen to produce chimeric vWD and normal pigs with and without platelet vWF, respectively. Analysis of plasma and platelets from these chimeric pigs confirmed that the crossed bone-marrow transplantation strategy was successful at modulating platelet vWF expression *in vivo*. Chimeric normal pigs expressed no platelet vWF, readily developed thrombosis, and exhibited partially corrected bleeding times. Conversely, chimeric vWD pigs expressed normal platelet vWF that was releasable at sites of exposed subendothelium but was not able to support thrombosis or correct prolonged bleeding times. Thus, vWF in the plasma compartment, endogenously produced, is essential for supporting thrombosis and, to a large extent, for correcting prolonged bleeding time.

It is interesting to note that vWD pigs with normal platelet vWF did not develop thrombosis or exhibit shortened bleeding

Table 3. Influence of infused plasma vWF on bleeding time and arterial thrombosis in chimeric vWD pigs expressing normal platelet vWF

Pig no.	vWF infusate, units/kg	Plasma		Bleeding time, min	Thrombosis per no. of arteries*
		vWF activity, %	vWF Ag, %		
95136	31.8	72	25	>15	0/2
95149	27.6	30	30	>15	0/2
05087	73.7	175	150	>15	0/2
				>15	0/6

*At baseline, none of seven arteries developed thrombosis (Table 2). When thrombosis did not occur, three of the four pigs were then infused with porcine vWF and monitored for an additional 60–120 min. In addition, the stenosis and injury protocol was performed on a separate artery in pig 05087 following vWF infusion. Bleeding time determinations were repeated at 30-min intervals during the stenosis and injury experimental procedure (3 hr).

times when infused with porcine vWF. In a series of infusions studies with vWD pigs (including the present study), we have achieved a wide range of vWF activity (30–175%), with a complete range of multimers (23, 26, 34). These plasma infusates did not support bleeding time or thrombosis. Bowie *et al.* (5) also noted a prolonged bleeding time in a vWD pig transplanted with normal marrow but found shortening of the bleeding time following vWF infusion. This apparent discrepancy could be due to differences in the infused products. Bowie *et al.* gave porcine cryoprecipitate and partially purified porcine vWF, while we infused chimeric vWD pigs with a porcine vWF product that had been concentrated in a process that separates porcine F.VIII. It is possible that processing plasma vWF alters the protein in such a way that it is not able to support thrombosis as does endogenous or native vWF. Alternatively, vWF in the subendothelium may be important for the correction of bleeding time or for the support of thrombosis. Further work is needed to clarify these issues.

The bleeding time was slightly prolonged in the normal pigs that had no platelet vWF. These data suggest that plasma vWF is a major determinant of bleeding time but that platelet vWF may also help correct prolonged bleeding times in pigs. Solberg *et al.* (29) found the bleeding time was prolonged in a normal pig with vWD marrow. This pig had no detectable platelet vWF by immunofluorescence and plasma vWF ristocetin cofactor activity of 10–20%. In humans, platelet vWF is also an important determinant of bleeding time (27, 30–32). In dogs that do not express platelet vWF, the bleeding time appears to be largely determined by plasma vWF (26). Thus, there may be species specificity of platelet vWF function as it relates to bleeding time. In pigs and humans, however, the vWF in the two compartments may interact for total correction of bleeding time, possibly by local delivery of high concentrations of vWF following platelet activation and release.

Bone marrow transplantation between normal and vWD pigs produced animals that had vWF only in plasma or platelets. Recent work has identified portions of the human vWF gene that are responsible for endothelial cell-specific expression of vWF (58, 59). To date, no specific regulatory elements in the human vWF gene have been identified that drive expression of vWF in megakaryocytes and ultimately platelet α granules. Since dogs appear to express vWF normally in their plasma but not in their platelets (26), 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.

vWF exhibits complex and diverse roles in thrombosis and hemostasis. Its function is determined in part by its compartmentalization, and it varies among species. Comparative studies have shown the essential contribution to arterial thrombosis

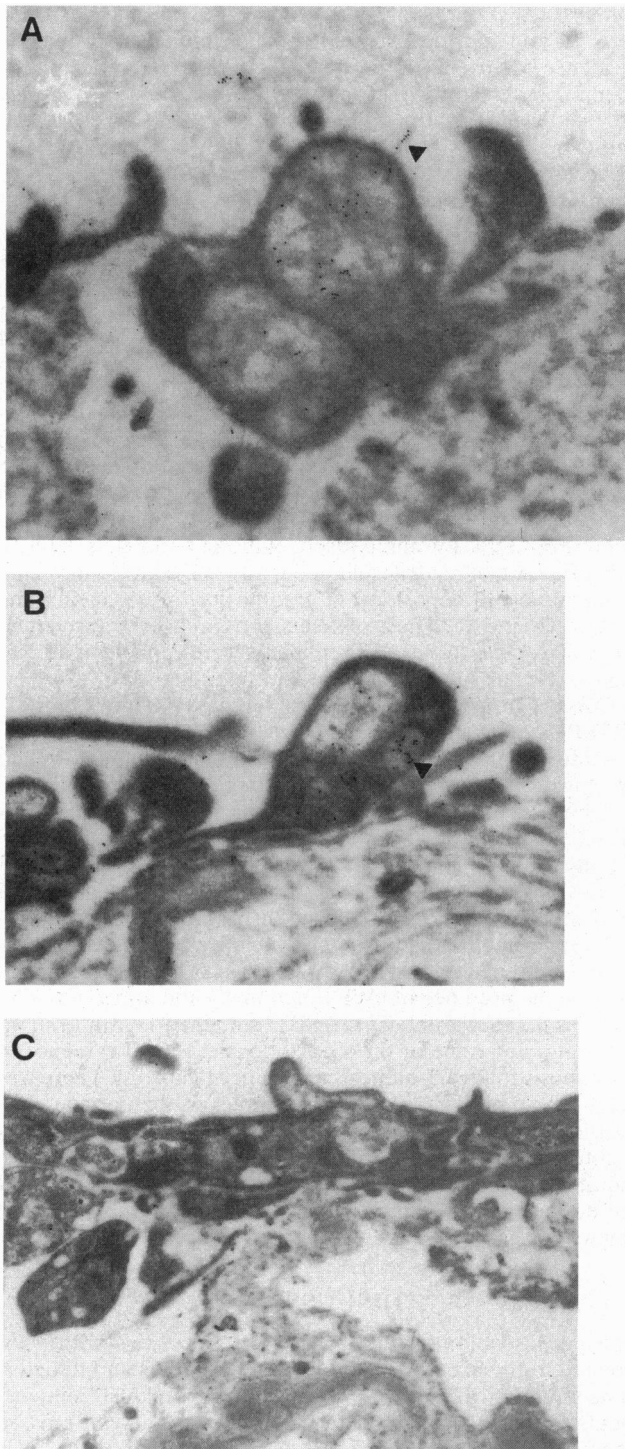


FIG. 3. Immunogold labeling of platelet vWF reveals the presence (arrowheads) or absence of specific antibody binding in platelet granules and on the surface of the adherent platelets. (A) TEM of normal platelets in a chimeric vWD pig ($\times 22,500$). Platelets are adhering to a vWD arterial subendothelium that had been perfused with blood from the transplanted pig in an annular perfusion chamber. Labeling of the platelet surface and granular material on platelets has been attributed to multimers of vWF (56, 57). (B) TEM of normal platelets in a normal pig ($\times 21,700$). Platelets are adhering to a normal pig arterial subendothelium that had been perfused with normal pig blood in an annular perfusion chamber. (C) TEM of vWD platelets from a chimeric normal pig ($\times 23,550$). The vWD platelets are adhering to normal pig subendothelium that had been perfused with blood from the transplanted pig in an annular perfusion chamber. No immunogold labeling for vWF in the adherent platelets is detected.

of vWF in the plasma compartments of pigs and dogs and have also suggested an important role of plasma and platelet vWF in bleeding time correction or hemostasis in pigs and humans.

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