THROMBOSIS RESEARCH 57; 109-116, 1990
0049-3848/90 $3.00 + .00 Printed in the USA.

VON WILLEBRAND FACTOR IS PRESENT IN THE VASCULAR ENDOTHELIUM FROM NORMAL DOGS AND FROM DOBERMAN PINSCHER DOGS WITH A PLASMA VON WILLEBRAND FACTOR DEFICIENCY

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(Received 2.1.1989; accepted in original form 23.8.1989 by Editor J.H. Griffin)

(Received in final form by Executive Editorial Office 24.10.1989)

ABSTRACT

An immunohistochemical study was undertaken to determine the presence and distribution of von Willebrand factor antigen (vWf:Ag) in blood vessels from normal dogs and from Doberman pinscher dogs with a marked plasma deficiency of vWf. vWf:Ag could not be detected in plasma from the Doberman pinscher dogs by ristocetin- and botrocetin-induced platelet agglutination or by EIA. An ELISA assay revealed vWf:Ag levels that were between 2-4% of that in normal canine plasma. Factor VIII:C activity was 30-46% of normal. The activated partial thromboplastin time (APTT) was increased but not the one-stage prothrombin time (OSPT). Four different antibody preparations were used in this study to detect vWf--a monoclonal and a polyclonal antibody prepared against human vWf and 2 polyclonal antibodies against canine vWf. vWf:Ag was detected with monospecific antibody in endothelial cells in veins, venules, and arterioles from normal dogs and vWf-deficient dogs. The histofluorescence observed in vessels of vWf-deficient dogs was indistinguishable from that observed in vessels from normal dogs.

INTRODUCTION

Von Willebrand factor (vWf) is a large glycoprotein of complex multimeric structure (1) that plays an important role in the adhesion of platelets to the subendothelium at high shear forces (2). Human vWf is synthesized in endothelial cells (3) and megakaryocytes (MK) (4) and circulates in plasma and platelets (5,6).

Von Willebrand's disease (VWD) is a hemorrhagic disorder caused by a deficiency of physiologically active plasma vWf. Three types of human VWD have been described (7,8). In Type I VWD all multimers are present but reduced. Type I has been subtyped depending on whether all multimers are reduced equally, platelet vWf is deficient, the multimers are

Key words: Von Willebrand Disease, canine, endothelium, immunohistofluorescence.

structurally altered, or plasma vWf increases after infusion of the vasopressin analogue, DDAVP. In Type II VWD the larger multimers are absent and in Type III VWD vWf cannot be detected. VWD is considered an endothelial disease by most investigators, but, due in part to ethical considerations, information on the synthesis and release of vWf by endothelial cells from VWD patients is scarce.

VWD has also been reported in pigs (9) and dogs (10). Certain breeds of dogs, such as the Doberman pinscher breed, have a very high incidence (11). It has been estimated that about 60% of the Doberman pinschers in North America have a vWf-deficiency (11). Dogs with VWD could serve as a resource for better understanding the endothelial processing of vWf and human VWD. This report describes an immunohistofluorescent study undertaken to determine the presence and distribution of vWf:Ag in biopsied blood vessels from normal and vWf-deficient dogs.

Since this report presents our initial characterization of Doberman pinschers with a marked plasma vWf deficiency, selected hemostatic parameters are presented.

MATERIALS AND METHODS

Animals and blood collection: Four vWf-deficient and 9 normal dogs (2 mongrels and 7 Labrador retrievers) were used. Hypothyroidism may be associated with decreased plasma vWf in humans (12) and dogs (10); the vWf-deficient Doberman pinschers in this study were euthyroid.

Pertinent hemostatic characteristics of vWf-deficient dogs were evaluated. Tests of hemostasis included: OSPT (13), APTT (13), plasma vWf:Ag measured by electroimmunoassay (EIA) (14) and an ELISA procedure, ristocetin cofactor (15), botrocetin agglutinating activity, bleeding time (16), glass-bead platelet retention (Thrombo-Screen, Sienco Inc., Morrison, CO), and factor VIII:C activity (17). Individual blood samples from the vWf-deficient dogs and the normal dogs were collected, via jugular venapuncture, into a syringe containing 0.11 M trisodium citrate at a ratio of 1 part citrate: 9 parts blood. The samples were kept on ice until platelet-poor plasma (PPP) was obtained by centrifuging the blood at 2,000 g for 20 min at 4°C. PPP was frozen at -70°C and analyzed within 4 weeks of procurement. This protocol was also used to obtain a normal plasma pool (NPP) from 20 mixed breed dogs by pooling and then freezing 10 ml of PPP from each dog. The vWf present in the NPP was 1 U/ml. Dr. W. Jean Dodds kindly supplied vWf-deficient plasma from a Scottish terrier dog that was homozygous for VWD. Cryoprecipitate from canine plasma was prepared as described by Zimmerman and Roberts (18).

Immunofluorescent detection of VIIIR:AG in vessels: The control and vWf-deficient dogs were anesthetized with thiamylal sodium (Biotal, Bio-Ceutic Lab, St. Louis, MO) and maintained on methoxyflurane (Metafane, Pitman-Moore, Washington Crossing, NJ). A segment of the caudal branch of the medial saphenous vein with accompanying arterioles and venules was surgically removed and the vessels rinsed with phosphate buffered saline (PBS) (0.15 M NaCl, 10 mM Phosphate, pH 7.4) at room temperature and then placed into Optimum Cutting Temperature compound (Miles Lab, Naperville, IL). The vessels were frozen immediately in liquid N_2 and stored at -70°C until 4 mm sections were made on a Miles Cryostat II. The sections were air dried, fixed in ethanol at -20°C for 0.5 min and then in acetone at -20°C for 2 min. The sections were then incubated with either a mouse monoclonal antibody to human vWf (1:100, Cappel Labs., Malvern, PA), rabbit antiserum to human vWf (1:100, Behring Dia., La Jolla, CA), 2 different rabbit antiserum to canine vWf (1:1000), an irrelevant monoclonal antibody, or rabbit preimmune serum for 30 min in a moist chamber. One antiserum to canine vWf was kindly supplied by Dr. W. Jean Dodds, while the other was prepared as described by Zimmerman and Roberts (18). Both antibodies were absorbed against vWf-deficient plasma. The irrelevant monoclonal antibody was against an anaplasmosis surface antigen and was supplied by Dr. William Davis. The antibodies were diluted in PBS containing 10% fetal calf serum. After washing with PBS the second antibody (1:1000), conjugated to fluorescein (USB, Cleveland, OH), rhodamine (Cappel Labs., Malvern, PA), or biotin (Vector Labs, Burlingame, CA), was applied to the slide and the slide incubated for 30 min. When biotin was used, a third incubation with rhodamine or fluorescein conjugated to avidin (1:1000, Cappel Labs, Malvern, PA) was performed.

Coverslips were mounted with buffered glycerol containing *p*-phenylenediamine (19) (Sigma Chemical Co., St. Louis, MO) and the slides were viewed and photographed on an Olympus BH-2 microscope.

Antibody absorption: Rabbit antiserum (1 ml) containing 0.2 mg of soybean trypsin inhibitor was incubated with 100 ul of canine plasma from vWf-deficient dogs that had been diluted with an equal volume of PBS at 37°C for 2 hrs while being constantly mixed. The plasma was placed at 4°C overnight and then centrifuged at 23,000 g for 30 min.

Crossed immunoelectrophoresis: The antigen was electrophoresed in 0.7% agarose in a tris barbital buffer (TBB) (Gelman Sciences High Resolution Buffer, #51104) for 3 hrs at 25 mA at 4°C. A strip of agarose containing the electrophoresed cryoprecipitate was placed on a glass plate covered with gel bond, and 0.7% agar in TBB (containing the appropriate antibody) was poured onto the plate. After cooling, the plate was electrophoresed for 16 hrs at 5 mA/plate at 4°C. The gels were rinsed for 16 hrs in 0.9% NaCl, stained for 30 min with Coomassie blue, and destained for 2-3 min in 10% acetic acid.

Immunodot: The immunodot assay was performed using nitrocellulose membranes in a Bio-Dot (Bio-Rad Lab., Richmond, CA) microfiltration apparatus according to the manufacturer's instructions.

ELISA: Monoclonal antibody (1:100) against human vWf (Cappel Labs., Malvern, PA), 50 ul, in 15 mM Na_2C0_3/35 mM $NaHCO_3$ was added to 96-well Costar flat bottom EIA plates (#3590) and incubated in a moist chamber at 15°C for 16 hrs. PBS containing 3% bovine serum albumin (BSA) was added as a blocking reagent and then removed after 30 min. Dilutions of the control canine pool or unknown samples, 50 ul, were added in PBS containing 1% BSA. One hr later the plates were washed 6 times with PBS containing 0.5M NaCl and 0.05% Tween-20. Monospecific rabbit antisera prepared against canine vWf (1:2000) was added and the plates were incubated for 1 hr. Another 30 min block was performed using PBS containing 3% BSA and preimmune goat serum (1:30). This was followed by an addition of Goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (1:1000) (Bio-Rad, Richman, CA) in PBS containing 1% BSA and preimmune goat serum (1:30). After 1 hr of incubation *o*-phenylenediamine (40 mg % in citrate-phosphate buffer containing 10 ul of 30% H_20_2/20 ml) was added and the optical density determined after a 1-hr incubation at a wave length of 450 nm. After the addition of each antibody, the plates were washed 6 times with PBS. The coefficient of variation for a plasma vWf:Ag concentration of 0.015 u/ml was 6.6%. The lower limit of detection was a plasma vWf:Ag concentration that was twice the background optical density, which corresponded to a plasma vWf:Ag concentration of 0.0025 u/ml.

Platelet agglutination using botrocetin: 20 ul of botrocetin (15 ug/ml, Biotoxin, Inc., St. Cloud, FL), lyophilized human platelets (1,000,000/ul, Helena Labs., Beaumont, TX) and the test sample were mixed on a mirrored surface and after 3 min of constant agitation the presence or absence of platelet agglutination was determined visually. In addition to the test sample, serial dilutions of control laboratory canine plasma pool made with 1% canine albumin in PBS were evaluated.

RESULTS

Hemostatic characteristics: The Doberman pinscher dogs had markedly reduced plasma vWf; it was below levels detectable by ristocetin, botrocetin and EIA (Table 1). vWf was present in plasma and could be detected with the more sensitive ELISA procedure at less than 0.05 U/ml. Platelet function tests requiring proper platelet adhesion were altered. Bleeding time was increased to between 4.5 and 7 min and glass-bead platelet retention was less than 50%. Coagulation was also affected. Factor VIII:C activity was less than 6% of the normal plasma pool. Correspondingly, the APTT was more than 1.5 sec longer than in the control dogs. The OSPT was not affected.

Antibody specificity: Four different antibody preparations were used to detect vWf:Ag in blood vessels from vWf-deficient dogs--a monoclonal antibody and polyclonal antibody prepared against human vWf and two polyclonal antibodies against canine vWf. The characteristics of one of the polyclonal antibodies prepared against canine vWf (obtained from Dr. W. Jean Dodds) has been reported previously (20). The second polyclonal antibody was

TABLE 1

Selected Hemostatic Parameters of Dogs Used in this Study.

Parameter	DOG vWf-deficient Doberman pinscher dogs* Fl	Fa	Fr	Me	Control dogs
vWf:Ag (U/ml)					
EIA	<0.1	<0.1	<0.1	<0.1	0.6-1.2
ELISA	0.03	0.02	.04	0.2	ND
Ristocetin Cofactor	<.15	<.15	.15	<.15	>.15
Botrocetin agglutinating activity (U/ml)	<.12	<.12	<.12	<.12	>.60
Bleeding time (min)	5	7	6.5	4.5	1.5-2.5
Glass-bead platelet retention (%)	25	35	35	49	>65
VIII:C (%)	32	30	46	42	55-108
OSPT (sec)	6.9	6.8	7.7	7.2	6.5-7.3
APTT (sec)	13.5	13.4	13.8	15.1	11.0-11.9

* Data represent mean of 3 determinations

prepared against canine vWf using vWf isolated from cryoprecipitates by gel filtration as described by Zimmerman and Roberts (18). Based on specific activities and following the calculations of Zimmerman and Roberts (18) the antigen used to inoculate the rabbits was enriched 24,000-fold in canine vWf.

The second antibody was monospecific since only single precipitation lines were observed in Ochterlony plates (Data not shown), and in crossed immunoelectrophoresis, only one precipitant line was visible as the concentration of the antibody (Data not shown) was increased. Furthermore, only one precipitation line was visible in crossed immunoelectrophoresis when the concentration of antigen, which was cryoprecipitate from control dogs, was changed from 0.25-2.5 mg/ml and the concentration of the antibody was kept constant (Data not shown).

The monospecificity of the second antibody was to vWf. The antibody did not react in Ochterlony plates, EIA, crossed immunoelectrophoresis or in the ELISA with plasma from a Scottish terrier dog that is homozygous for VWD. This homozygous vWf-deficient plasma did not support botrocetin-induced platelet agglutination.

The commercially obtained polyclonal antibody to human vWf cross-reacted with canine vWf, as indicated by lines of identity, with antisera to canine vWf in Ochterlony plates (Data not shown). The monoclonal specificity was determined in an immunodot assay. A positive reaction was observed with control canine pool plasma, but not with plasma from a dog that is homozygous for vWd.

Immunological detection of vWf:Ag in canine blood vessels: Each antibody detected vWf:Ag in veins, venules, and arterioles from the control and the vWf-deficient dogs; in fact, there was no detectable difference in the localization of vWf:Ag or the fluorescence intensity in vessels from control dogs and vWf-deficient dogs (Fig. 1).

Immunofluorescence was observed only in areas of the vessel occupied by endothelial cells. For example, in arterioles immunofluorescence could be seen only in the tunica media between the internal elastic membrane and the lumen; in veins, fluorescence was detected only adjacent to the lumen (Fig. 1). vWf:Ag had a punctate distribution and was not evenly distributed within the endothelium (Fig. 1).

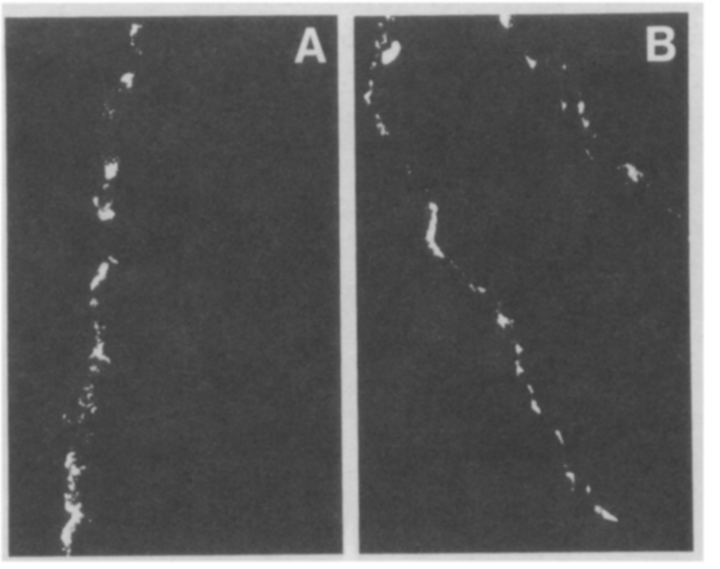

Fig. 1. Immunohistofluorescent micrographs of a segment of the caudal branch of the medial saphenous vein from a vWf-deficient Doberman pinscher dog (A) and control dog (B) showing the presence of vascular VIIIR:Ag in veins from both dogs. VIIIR:Ag was detected using a mouse monoclonal antibody against human vWf as described in the Materials and Methods section.

The immunofluorescence appeared specific for vWf:Ag since endothelial immunofluorescence was not observed when an irrelevant monoclonal antibody or preimmune antiserum was used. Furthermore, absorption with control plasma, but not vWf-deficient plasma, eliminated endothelial cell reactions.

DISCUSSION

In the Doberman pinscher dogs bleeding time was prolonged and glass bead platelet retention was reduced. This is associated with a marked reduction in vWf:Ag, which accounts for the absence in ristocetin- and botrocetin-induced platelet agglutination. A marked reduction in plasma vWf (<0.1 U/ml) has been previously reported for Doberman pinschers (10,21). There are also Doberman pinschers with intermediate levels of vWf:Ag that vary between the marked deficiency reported here and normal values (10). Because the inheritance pattern of VWD in Doberman pinschers has not been established and the hormonal status, including thyroid function, was often not evaluated, the relationship of dogs with intermittent levels to those that are more markedly deficient is not understood.

Reduced canine factor VIII:C activity with a prolongation of the APTT was observed. Reduced factor VIII:C activity is also seen in human VWD (22) and porcine VWD (9). The decrease in factor VIII:C in the 3 species with VWD is less than vWf:Ag, supporting the hypothesis that reduced factor VIII:C activity is associated with VWD, but it is secondary to the vWf deficiency (22).

Potter et al. (23) could not demonstrate vWf:Ag with immunofluorescence in canine endothelial cells. The difference between the 2 reports may be in the fixation procedure since, in our hands, antigenicity was lost when their procedure was followed. Potter et al. (23) also could not demonstrate vWf:Ag in canine platelets. Using the same antibodies and dogs that were involved in this study, Meyers et al. (24) could not demonstrate vWf:Ag in platelets or megakaryocytes from the normal or vWf-deficient dogs. On the other hand, vWf:Ag could readily be identified in human, bovine, equine, and feline platelets. These findings suggest that even though plasma vWf and platelet vWf are reduced in the vWf-deficient dogs, the inability to detect platelet vWf may not be related to VWD.

It is common to find dogs similar to those in this study who have never experienced an episode of clinical bleeding following trauma or minor surgical procedures such as cosmetic otoplasty (25). Bleeding times in the vWf-deficient dogs in this study were not as prolonged as they are in animals with qualitative platelet defects (26,27). Therefore, it appears that the hemostatic impairment does not correlate with the plasma vWf deficiency. If it is assumed that vWf is necessary for platelet to adhere to the subendothelium, then vWf other than plasma vWf may be participating in hemostasis. The inability to detect vWf in canine platelets (24) suggests that platelet vWf cannot be contributing significantly to hemostasis. The presence of vWf:Ag in the endothelium of vWf-deficient dogs indicates that vWf could be released locally from the endothelium in response to trauma. If dogs are similar to humans, the vWf released from the endothelium will have larger multimers (28) which may be more physiologically active (29) and may account, in part, for the hemostatic competency of vWf-deficient Doberman pinscher dogs.

Information on the synthesis and release of vWf from endothelial cells in patients with VWD is lacking since the hemorrhagic tendency, and the unacceptable risks associated with supplement therapy, preclude obtaining blood vessels from VWD patients. The distribution of vWf in endothelial cells from VWD patients has been described only a few times. vWf:Ag was not found in endothelial cells from patients with severe autosomal recessive VWD (30), but was found in endothelial cells from a patient whose plasma level of vWf:Ag was 5% of normal (31) and in a patient with moderately severe Type I VWD (30). The distribution of vWf in the former patient is, therefore, similar to porcine VWD (32) where vWf cannot be identified by immunofluorescence in biopsied vessels while the latter patient with Type I VWD is similar to the vWf-deficient Doberman pinscher dogs used in this study. The observation that the vWf-deficient Doberman pinscher dogs have endothelial vWf:Ag, suggests that these dogs could be a useful model to study the synthesis, processing, storage and release of endothelial vWf.

ACKNOWLEDGEMENTS

This study was supported in part by grants, RR-00191, HL 02146, and HL 37485 from the National Institutes of Health.

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