Molecular and genetic basis for thrombasthenic thrombopathia in Otterhounds

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Objectives—To determine the molecular and genetic basis for thrombasthenic thrombopathia in Otterhounds and establish whether the defect would be best classified as type-I Glanzmann's thrombasthenia.

Animals—57 dogs, including 13 affected Otterhounds, 23 carrier Otterhounds, 17 unaffected Otterhounds, and 4 clinically normal unrelated dogs of other breeds.

Procedure—Functional (platelet aggregation, clot retraction, buccal mucosa bleeding time) and biochemical (electrophoresis, flow cytometry, fibrinogen content) analyses were conducted. In addition, first-strand cDNA synthesis from platelet total RNA was performed. Exons of the genes encoding for glycoproteins (GP) IIb and IIIa were amplified in overlapping fashion. The resulting products were excised from agarose gels and sequenced. The sequences obtained were compared with known cDNA sequences for canine GPIIb and GPIIIa.

Results—A single nucleotide change at position G1193 (1100) was detected in exon 12 of the gene encoding for platelet GPIIb in 2 affected Otterhounds. Carrier Otterhounds were heterozygous at this position, and 2 unaffected Otterhounds were unchanged. This nucleotide change would result in substitution of histidine for aspartic acid at position 398 (367) within the third calcium-binding domain of GPIIb.

Conclusions and Clinical Relevance—These studies suggest that thrombasthenic thrombopathia of Otterhounds is homologous phenotypically and has a similar molecular basis to type-I Glanzmann's thrombasthenia in humans. (*Am J Vet Res* 2001;62:1797–1804)

An autosomal recessive inherited intrinsic functional disorder of platelets in humans was initially described in 1918 by a Swiss pediatrician.[1] The disease, known as **Glanzmann's thrombasthenia (GT)**, is characterized by failure of platelets to aggregate in response to a number of agonists including ADP, collagen, thrombin, and platelet-activating factor; abnormal clot retraction, diminished or undetectable amounts of **glycoproteins (GP)** IIb and IIIa on platelet membranes; and decreased fibrinogen content in platelet α granules. Clinical signs consist primarily of mucosal bleeding including epistaxis, gingival bleeding, and petechial and ecchymotic hemorrhages. Bleeding episodes may be mild to severe; the disease may be especially problematic in humans in situations such as tooth extractions, parturition, menstruation, surgery, or trauma.[2,3]

The GPIIb-IIIa complex (also known as integrin aIIbβ_3 or as the fibrinogen receptor) is responsible for mediating platelet aggregation via binding of the dimeric ligand fibrinogen.[4] This glycoprotein complex also mediates the uptake of plasma fibrinogen that is ultimately stored in platelet α granules.[5] Glycoproteins IIb and IIIa are products of 2 separate genes that have been mapped on the q21 to q23 band of chromosome 17 in humans.[6,7] Each subunit is synthesized as a separate protein and forms the complex within the endoplasmic reticulum. From there, the complex is transported to the Golgi apparatus where the IIb subunit is cleaved into heavy and light chains that are linked by disulfide bonds. The mature complex then is transported to the platelet surface. Both subunits are required for effective formation of the complex and maintenance of a stable GPIIb-IIIa complex on the platelet surface.[8] Mutations in either of the genes encoding for IIb or IIIa can result in ineffective formation of the GPIIb-IIIa complex.[3] A molecular mechanism was first described in an affected human in 1990.[9] Since that time, > 50 mutations have been described in the genes encoding for GPIIb and GPIIIa that can cause GT in humans.[10]

Three categories of GT are recognized.[2] Type-I GT is characterized by platelets that have < 5% of the normal concentration of GPIIb and GPIIIa on the platelet surface, abnormal clot retraction, severe impairment of platelet aggregation responses, and reduced to undetectable amounts of fibrinogen in platelet α granules. More cases of type-I GT are attributed to defects in the gene encoding for GPIIb than the gene for GPIIIa.[10] Type-II GT is characterized by platelets that have 10 to 20% of the normal concentration of GPIIb and GPIIIa on the platelet surface. Platelet aggregation responses, clot retraction, and fibrinogen content in platelet α granules may be moderately or severely impaired or reduced. Variant GT is a qualitative disorder of the fi brinogen receptor. Glycoprotein IIIb-IIIa complexes may be evident in platelets at 50 to 100% of their normal concentration, but function is severely impaired. Most cases of type-II and variant GT in humans are the results of defects in the gene encoding for GPIIIa.[10] An interesting observation in people affected with GT as a result of a defect in the gene encoding for GPIIb is that increased amounts of the vitronectin receptor are found on surface of platelets.[11,12] This phenomenon is believed to be the result of increased availability of the β_3 subunit for binding to α vitronectin subunits,

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because GPIIb subunits are unavailable or too unstable to form complexes. The use of flow cytometry for detection of increased amounts of the vitronectin receptor or detectable amounts of GPIIIa on the surface of platelets from humans with type-I GT should direct investigators to consider that the gene encoding for GPIIb is the most likely location of the defect.

The molecular basis for type-I GT in dogs was first described in Great Pyrenees dogs in 1999[13]; however, clinical aspects, platelet function, and biochemical data for this defect were confirmed in 1996.[14] Mucosal bleeding, severe impairment of platelet aggregation responses, severe reduction in fibrinogen content of platelets, lack of clot retraction, and severe reduction in amounts of GPIIb and GPIIIa from the platelet surface unequivocally confirm a diagnosis of type-I GT. Because the genes encoding for platelet GPIIb and GPIIIa were not sequenced in dogs until 1999, the defect could not be characterized further for the dogs in 1996. When the 2 genes were sequenced in 1999, the defect was determined to be within the gene encoding for GPIIb.[13,15]

Thrombasthenic thrombopathia of Otterhounds was first recognized in 1967.[16] Affected dogs have mucosal bleeding and prolonged bleeding times. Platelet aggregation responses in response to all agonists are minimal or lacking, clot retraction is severely impaired, and intraplatelet fibrinogen content is severely reduced. There is a reduction of GPIIb-IIIa complexes on the platelet surface. In early studies, some affected Otterhounds were reported to have large platelets with a bizarre morphology not characteristic of GT. This observation raised the possibility that the defect was a combination of a platelet-function disorder with features of GT and Bernard Soulier's disease.[17] Bernard Soulier's disease is an inherited intrinsic defect in platelet function attributable to a defective platelet complex GPIb-IX-V.[18] Four genes encode the 4 protein subunits of the complex. Platelets with that defect have an abnormal adhesion mechanism, but platelet aggregation responses are normal. Morphologically, platelets are described as large and bizarre in appearance.

Abnormal clot retraction has been used as a screening test for thrombasthenic thrombopathia, and wide-scale use of this technique by Otterhound breeders led to virtual elimination of the defect. However, in the early 1990s, descendants of dogs originally described as affected by thrombasthenic thrombopathia were reportedly affected; these descendants had a severe bleeding disorder associated with abnormal clot retraction. Platelets from affected dogs in this population had a severe reduction of aggregation in response to ADP, collagen, and thrombin. They had undetectable to trace amounts of platelet-membrane GPIIb and GPIIIa, accompanied by a severe reduction in intraplatelet fibrinogen content. Changes in other membrane glycoproteins were not detected. In contrast to earlier reports, differences in platelet size or morphologic characteristics were not observed. Evidence for a combined defect in platelet function was not obtained.

The objective of the study reported here was to sequence the cDNA encoding for GPIIb and GPIIIa in clinically normal (unaffected), carrier, and affected Otterhounds. In addition, we intended to establish whether thrombasthenic thrombopathia of Otterhounds was a form of type-I GT.

Materials and Methods

Animals and blood collection—Blood samples were obtained from 38 related Otterhounds and 27 unrelated Otterhounds referred for analysis of platelet function, using standard aggregometry methods.[19] Food was withheld for 12 hours from all tested dogs, and blood samples were collected from a cephalic vein into plastic syringes containing anticoagulant (9 parts blood:1 part 3.8% trisodium citrate [vol:vol]. Dogs included in the study had not received any medication for ≥ 10 days prior to collection of samples.

Platelet-rich plasma (PRP) was prepared at room temperature (21 C) by successively centrifuging citrated blood samples 3 times (3 minutes at 650 × g per centrifugation). The PRP fractions obtained after each centrifugation were pooled. **Platelet-poor plasma (PPP)** was obtained at room temperature (21 C) by centrifugation of blood samples at 2,200 × g for 15 minutes. For platelet aggregation studies, pooled PRP was adjusted by addition of autologous PPP to achieve a final concentration of 300,000 platelets/µl.

Gel-filtered platelets (GFP) were prepared from citrated PRP on a 1.5 × 30-cm siliconized glass column. The column was packed with acetone-washed agarose beads[a] and equilibrated with elution buffer (147 m*M* NaCl, 5 m*M* KCl, 0.05 m*M* $CaCl_2$, 0.1 m*M* $MgCl_2$, 5 m*M* HEPES), 5.5 m*M* glucose (pH 7.4), and 0.35% bovine serum albumin.[b] Five to 7 ml of citrated-PRP was applied to the top of the column and eluted with elution buffer until the platelet fraction was detected in the void volume of the column (approx 11 to 12 ml), as indicated by a change in eluate turbidity. Fractions (1 ml) then were collected into plastic tubes. The eluted fractions were pooled, and the number of platelets was determined. Platelet recovery exceeded 87%. The GFP were diluted with elution buffer to achieve a final concentration of 300,000 GFP/µl.

Platelet aggregation responses of clinically normal dogs and Otterhounds were evaluated by changes in the turbidity of PRP following addition of an agonist in a dual-channel aggregometer[c] equipped with a chart recorder. Baseline (0% platelet aggregation) value was established by use of PRP or GFP, whereas PPP or GFP-elution buffer was used to represent 100% platelet aggregation.

In a typical experiment, 0.45 ml of PRP (300,000 platelets/µl) was added to a 1-ml siliconized glass cuvette containing a plastic-coated stir bar. The PRP suspension was warmed to 37 C with gentle stirring (aggregometer setting of 850 revolutions/min), and 0.05 ml of the appropriate agonist was added. The extent of platelet aggregation was measured for 4 to 6 minutes following addition of the agonist. Changes in platelet shape were monitored photometrically.

The ability of Otterhound platelets to support clot retraction was evaluated in dilute samples of whole blood.[20] For each control and test dog, a 5-ml plastic syringe was filled with 4.5 ml of cold (2 to 8 C) saline (0.9% NaCl) solution and maintained at 2 to 8 C until used. An aliquot (0.5 ml) of blood was collected from a vein of each control and test dog into the syringes containing the chilled saline solution; each syringe was inverted gently several times 0to thoroughly mix each sample. Duplicate aliquots (2 ml) of this dilute blood sample were transferred to 10 × 75-mm glass tubes containing 0.1 ml of bovine thrombin[d] that had been diluted immediately prior to use with cold saline solution to achieve a final concentration of 10 U/ml. Tubes were covered with parafilm and inverted gently to ensure mixing. After mixing, tubes for the control and test dogs were transferred

to a water bath (37 C). The reaction was timed and scored for degree of clot retraction at intervals of 1, 2, and 4 hours. Tubes were assigned scores on a scale of 1 to 4+ (1 represented minimal retraction of the clot, and 4+ represented maximal retraction of the clot).

Tests were performed to determine buccal mucosal bleeding time, as described elsewhere.[21] Dogs were maintained in sternal recumbency during the procedure. The upper lip was everted and secured, using a 2-in wide strip of gauze wrapped snugly around the muzzle. A spring-loaded bleeding device[e] was triggered, producing 2 linear incisions in the buccal mucosa parallel to the lip margin. Blood flowing from the incisions was blotted at 5-second intervals on filter paper[f] placed directly below, but not touching, the wounds. Any fibrin film forming over the incision was gently dislodged. The time from triggering the device until blood was not apparent on the filter paper was recorded for each incision, and the mean was recorded as buccal mucosal bleeding time. When bleeding had not ceased by 15 minutes, direct pressure was applied to the incisions for 1 to 2 minutes. When bleeding persisted or recurred after it had initially ceased, a topical tissue adhesive[g] was applied.

Electrophoresis of platelet-membrane GP was performed on samples of washed platelets obtained from 5 clinically normal dogs, 2 unaffected Otterhounds, and 5 affected Otterhounds. The PRP was prepared for each dog from blood samples obtained at a ratio of 9 parts blood:1 part anticoagulant (vol:vol), using ACD (0.14*M* citric acid, 0.16*M* trisodium citrate, and 0.11*M* glucose [pH 4.3]) as the anticoagulant. Prostaglandin E_1 was added to the ACD to achieve a final concentration of 1 µ*M*. Platelets were washed 3 times by repeated centrifugation (2,500 × g for 10 minutes at 21 C) in Tris-buffered saline solution (pH 7.4) that contained glucose and EDTA (0.01*M* Tris, 0.15*M* NaCl, 0.005*M* glucose, and 0.001*M* EDTA). Detergent extraction of platelet-membrane proteins was achieved by resuspension of the final washed platelet pellets in 1% (vol:vol) Triton X-100 in Tris-glycine buffer (38 m*M* Tris, 100 m*M* glycine [pH 8.7]) followed by ultrasonication (3 times for 5 s/ultrasonication at 4 C), using a homogenizer[h] at a setting of 8.

Protein extracts were solubilized and reduced by incubation in a solution of 2% **sodium dodecyl sulfate (SDS)** containing 10 m*M* Tris, 150 m*M* NaCl, 3 m*M* EDTA, and 5 m*M* N-ethylmaleimide and 5% (vol:vol) 2-mercaptoethanol at 37 C for 1 hour. Samples then were applied to a polyacrylamide gel. Samples containing 100 to 400 µg of protein were analyzed on 6% polyacrylamide Laemmli slab gels overlaid with a 3.5% stacking gel, electrophoresed, and stained with periodic-acid Schiff reagent.[19] Orosomucoid (5 µg/sample) was used as an internal GP standard. Gels were scanned, using a scanning spectrophotometer[i] set at 550 nm.

Fibrinogen content in platelets was determined in 7 affected dogs by use of an enzyme immunosorbent assay[19] or an ELISA.[22] For the ELISA, pooled plasma (n = 20 dogs) that had a fibrinogen concentration of 385 mg/dl was used for the standard. Standards and platelet extracts obtained by use of Triton X-100 were diluted in ELISA dilution buffer (0.9% NaCl, 0.05% bovine serum albumin,[j] 20 m*M* Tris, 3 m*M* sodium EDTA, pH 7.4). The assay was linear for canine fibrinogen concentrations of 14 to 380 ng/ml. Triton X-100 did not interfere with the assay. Capture, sandwich, and detector antibodies for the canine fibrinogen ELISA were formatted similar to the technique reported for rodent fibrinogen.[22]

Platelet volume was determined by personnel in the Clinical Pathology Laboratory in the Veterinary Teaching Hospital at Cornell University. Platelet volume was determined in 3.8% citrate-anticoagulated blood samples, using an automated hematology analyzer.[k]

Blood samples for flow cytometric analysis were collected into anticoagulant (ACD; NIH formula A). Platelets were subsequently isolated and washed, using the method of Thiagarajan and Tait.[23] Washed platelets were resuspended and diluted to a concentration of 1×10^7 cells/ml, as previously described.[24] Platelets then were probed for membrane GPIIIa, using a purified **fluorescein isothiocyanate (FITC)**-conjugated murine monoclonal antibody to human GPIIIa[l] or an isotype FITC-conjugated mouse IgG1 negative-control antibody.[m] In contrast to antibodies developed against human GPIIIa, commercially available antibodies to human GPIIb do not cross-react with canine platelet GPIIb. Thus, flow cytometric analysis could only be used to measure canine GPIIIa. Flow cytometric analysis was performed, using a flow cytometer[n] in accordance with manufacturer's protocols.

For total RNA, PRP was isolated via differential centrifugation from 50-ml aliquots of blood collected into EDTA from each of 2 affected, 1 carrier, and 2 unaffected Otterhounds. Prostaglandin E_1 (final concentration of 3 µ*M*) was added to PRP, platelets were centrifuged, and the pellet was resuspended in a small volume of plasma. Platelets were transferred to RNase-free tubes and centrifuged. The plasma supernatant was removed. Platelet pellets were frozen immediately at –70 C and shipped on dry ice via overnight courier to Auburn University. Total RNA was prepared, and first-strand cDNA synthesis was conducted on platelet samples, as described elsewhere.[25] Exons of the genes encoding for GPIIb and IIIa were amplified in overlapping fashion, using specific primers designed in other studies.[13,15,25] Once the location of the defect was defined, specific primers (forward, 5'-ATGGCGTCGTATTTTGGGCAT-3'; reverse, 5'-CATAAGCTCCTACCAGTAGGT-3') were used to amplify a segment between exons 11 and 14 in platelet-derived cDNA (469 base-pair fragment). The forward primer was based on a human sequence, whereas the reverse primer was based on a canine sequence. **Polymerase chain reactions (PCR)** to amplify the area of interest consisted of an initial denaturation step (4 minutes at 94 C) followed by 30 cycles (denaturation, 94 C for 60 seconds; annealing, 45 C for 60 seconds; and extension, 72 C for 2 minutes). A final extension (7 minutes at 72 C) was performed at the end of the reaction. The PCR products were electrophoresed and excised from 1.5% agarose gels, using a kit.[o] Purified amplified DNA products were submitted directly for sequencing. Evaluation of DNA sequence was performed on PCR products, using a commercially available kit.[p] Extension products were sequenced, using an automated DNA sequencer.[q]

Results

Fifty-three purebred Otterhounds were evaluated for GT, using results of platelet function and clot retraction. A multiple-generation pedigree documenting the autosomal inheritance of GT in 38 related purebred Otterhounds was created (**Fig 1**). Dogs ranged from 6 weeks to 8 years old (mean, 1.6 years). Five dogs (1 male, 4 females) were identified with severe GT on the basis that their platelet samples failed to aggregate in response to ADP (10 µ*M*) or collagen (12 µg/ml). Platelets from dogs with severe GT failed to support normal clot retraction. They had a dilute whole-blood clot retraction score of 1+ (reference range, 3 to 4+ on a scale of 1 to 4+). Typical dilute whole-blood clot retraction responses for platelets obtained from unaffected Otterhounds and Otterhounds with GT were compared (**Fig 2**).

Thirteen Otterhounds had intermediate and reversible platelet reactivity to 10 µ*M* ADP (range, 13 to 52% platelet aggregation; reference range, > 60% irre-

versible platelet aggregation). On the basis of the intermediate reactivity to ADP, they were classified as putative carriers for GT. In contrast, maximal platelet aggregation in response to addition of collagen (12 µg/ml) for the putative GT carriers (range, 63 to 98% platelet aggregation) was within the reference range. Clot retraction for the putative carriers ranged from 3 to 4+.

Of 27 Otterhounds screened for GT, 8 were identified with severe GT. Relationship of these dogs to the other 38 related Otterhounds was not established because of incomplete information about pedigrees. Most of the 27 dogs in this population were referred for evaluation because of a history of suspected or established bleeding disorders or because the owners wanted the puppies screened for GT at 6 to 12 weeks of age.

Clinical manifestations in Otterhounds with severe GT included hemorrhage of the gingival mucosa, epistaxis, melena, hematuria, cutaneous ecchymoses, and prolonged or excessive bleeding at sites of surgery or trauma. Clinical signs of bleeding disorders were recognized in some affected puppies as early as 4 weeks after birth, whereas bleeding problems in most Otterhounds with GT were recognized before they were 1 year old.

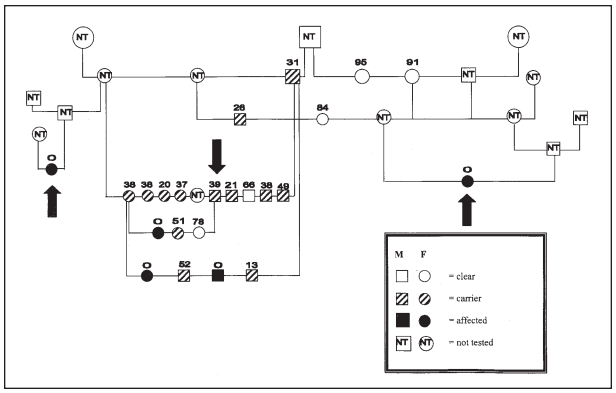

Figure 1—Multiple-generation pedigree of Otterhounds with type-I Glanzmann's thrombasthenia (GT). Unaffected, carrier, and affected status were determined on the basis of platelet function. Numbers above specific dogs indicate maximal percentage platelet aggregation in response to 10 µ*M* ADP. Three dogs were selected for molecular genetic analysis (black arrows).

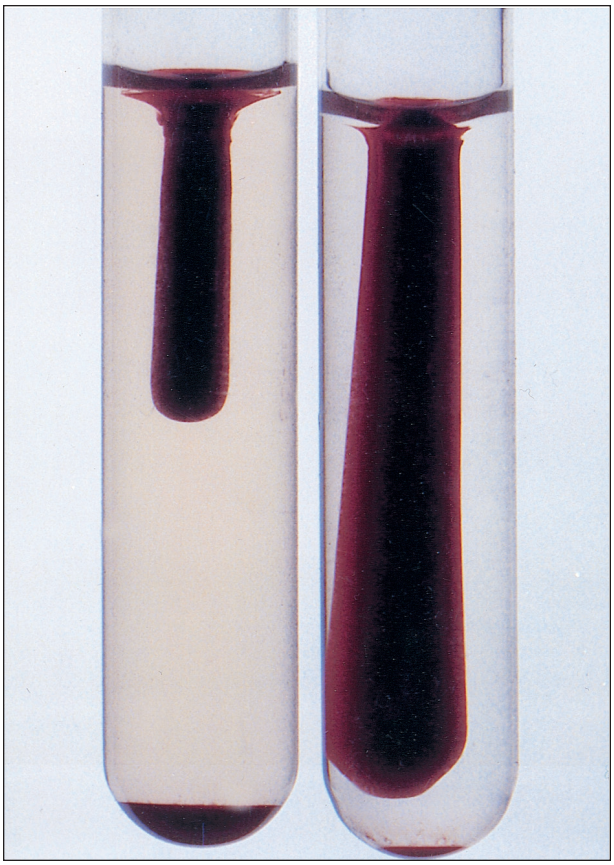

Figure 2—Photograph depicting dilute whole-blood clot retraction for samples obtained from an unaffected Otterhound (left tube) and an Otterhound with severe GT (right tube). Samples were stimulated with 0.2 U of bovine thrombin and allowed to incubate undisturbed for 4 hours at 37 C. Notice the failure (score of 1+, reference range, 3 to 4+) of the platelets of the GT-affected Otterhound to effect retraction of the clot.

Table 1—Results for functional and biochemical tests of platelets from Otterhounds* with thrombasthenic thrombopathia and reported reference ranges

Test	Affected	Reference range
BMBT	> 15 minutes	2 to 4 minutes
Platelet volume (n = 2)	8.3 and 10.6 fl	7 to 11 fl
Clot retraction	1+	3 to 4+
Platelet aggregation		
Platelet-rich plasma		
ADP (10–100 µ*M*)	Change in shape only	> 60% light transmittance
Collagen (12–100 µg/ml)	Change in shape only	> 60% light transmittance
Gel-filtered platelets		
α–thrombin (1 U/ml)	Change in shape only	> 60% light transmittance
Calcium ionophore		
A23187 (4–40 µ*M*)	Change in shape only	> 60% light transmittance
Electrophoresis of membrane glycoproteins (GP; n = 5)		
GPIIb	Undetectable	Normal
GPIIIa	Undetectable	Normal
GPIb	Normal	Normal
Intraplatelet fibrinogen concentration (n = 7)	0.21 ± 0.09 µg/10^8 platelets	6.5 ± 2.2 µg/10^8 platelets

*Values reported are for 13 dogs unless otherwise indicated.
BMBT = Buccal mucosal bleeding time.

Complications associated with uncontrolled bleeding during or after ovariohysterectomy or neutering were encountered in GT-affected Otterhounds, and these dogs often required transfusions.

Results of the initial screening tests performed in affected Otterhounds were summarized (**Table 1**). Analysis of platelet function verified the existence of a global defect in platelet aggregation in response to ADP, collagen, γ-thrombin, platelet-activating factor, and calcium ionophore A23187, as determined by comparing results for platelets obtained from unaffected and GT-affected Otterhounds (**Fig 3**). Similar results for each agonist were obtained when GFP were used (data not shown). Global platelet dysfunction in combination with abnormal clot retraction and a prolonged buccal mucosal bleeding time are cardinal features characteristic of type-I GT. Number and size of platelets from GT-affected Otterhounds were within reference ranges, and platelet morphology appeared normal during light microscopic examination. Consistent with another study,[17] platelet GPIIb and GPIIIa were not detectable or detectable only in trace amounts when evaluated on the basis of results for electrophoresis of platelet-membrane GP, whereas normal quantities of GPIb were detected (**Fig 4**). The trace amount of GPIIIa observed in the scan of the sample obtained from affected Otterhounds was not consistently detected. Trace amounts of platelet-membrane GP may be difficult to detect by use of periodic-acid Schiff staining if destaining fails to remove background stain. Specific identification of GPIIb and GPIIIa by use of western blotting techniques would have been attempted if antibodies to human GPIIb capable of cross-reaction with canine GPIIb would have been available.

To specifically identify GPIIIa on the surface of platelet membranes, flow cytometry was used. Washed platelets from a clinically normal dog and an affected thrombasthenic Otterhound were subjected to flow cytometry. When forward- and side-scatter plots were used to define size of platelets in both populations, we did not detect significant differences in size distribution between the affected Otterhound and the clinically normal dog (data not shown). Incubation of platelets from the clinically normal dog or the affected

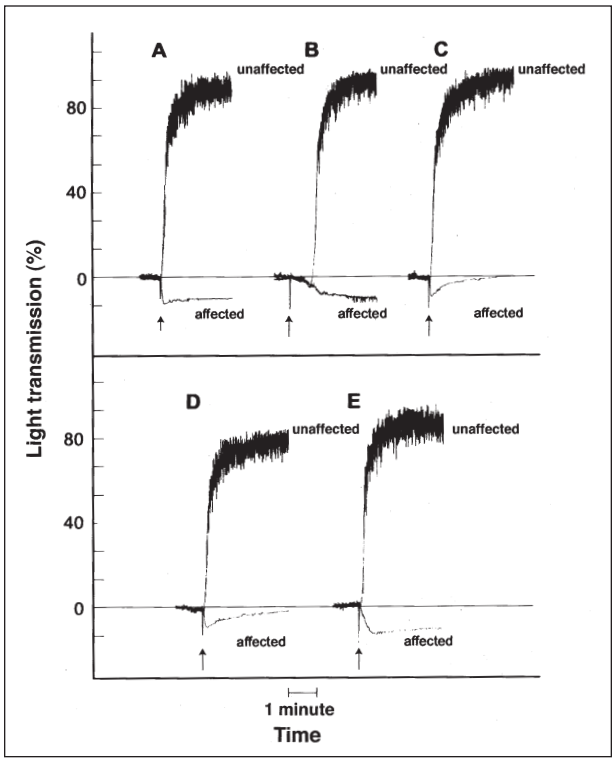

Figure 3—Platelet aggregation for samples obtained from unaffected and GT-affected Otterhounds. Platelet activation was initiated by addition (arrow) of 50 µl of each agonist (A, 10 µ*M* ADP; B, 12 µg of collagen/ml; C, 0.2 µ*M* γ-thrombin; D, 2 µ*M* platelet-activating factor; E, 10 µ*M* A23187) to citrated platelet-rich plasma (300,000 platelets/µl). Tracings are from a single experiment and are representative of results obtained for each severely affected Otterhound and an equal number of unaffected Otterhounds.

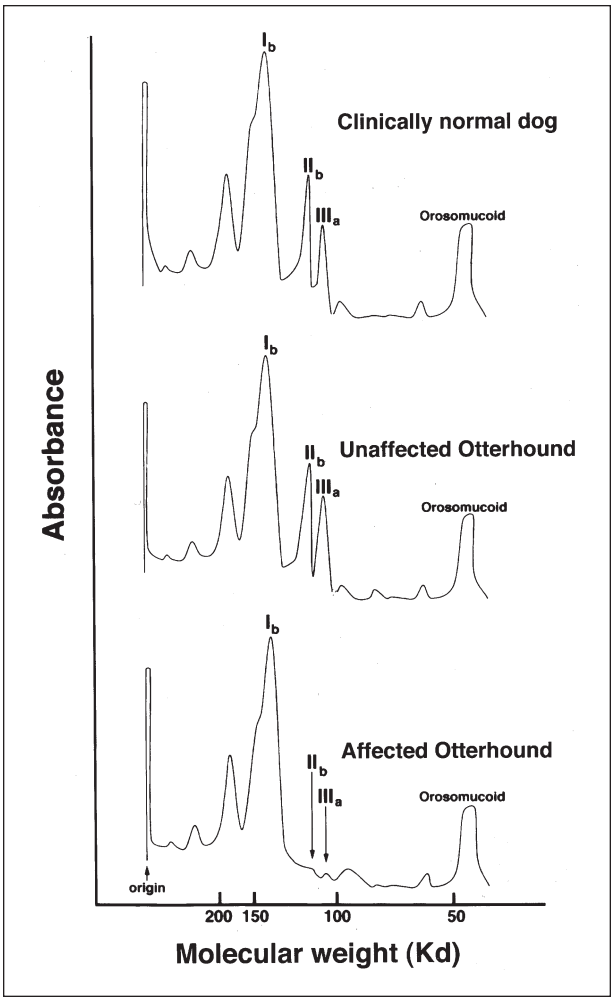

Figure 4—Densitometric scans of periodic-acid Schiff-reactive canine platelet-membrane glycoproteins (GP) in washed platelets from unaffected and GT-affected Otterhounds. Platelets were solubilized in 2% sodium dodecyl sulfate (SDS). Extracts then were reduced (5% 2-mercaptoethanol [vol:vol]) for 1 hour at 37 C, 400 µg of platelet protein was applied to each lane, and proteins were electrophoresed through a 6% polyacrylamide resolving slab gel. Results were scanned on a spectrophotometer set at 550 nm. Orosomucoid was included in each gel as a standard. Notice in the affected Otterhound scan the normal peak for GPIb and the marked reduction of peaks for GPIIb and GPIIIa.

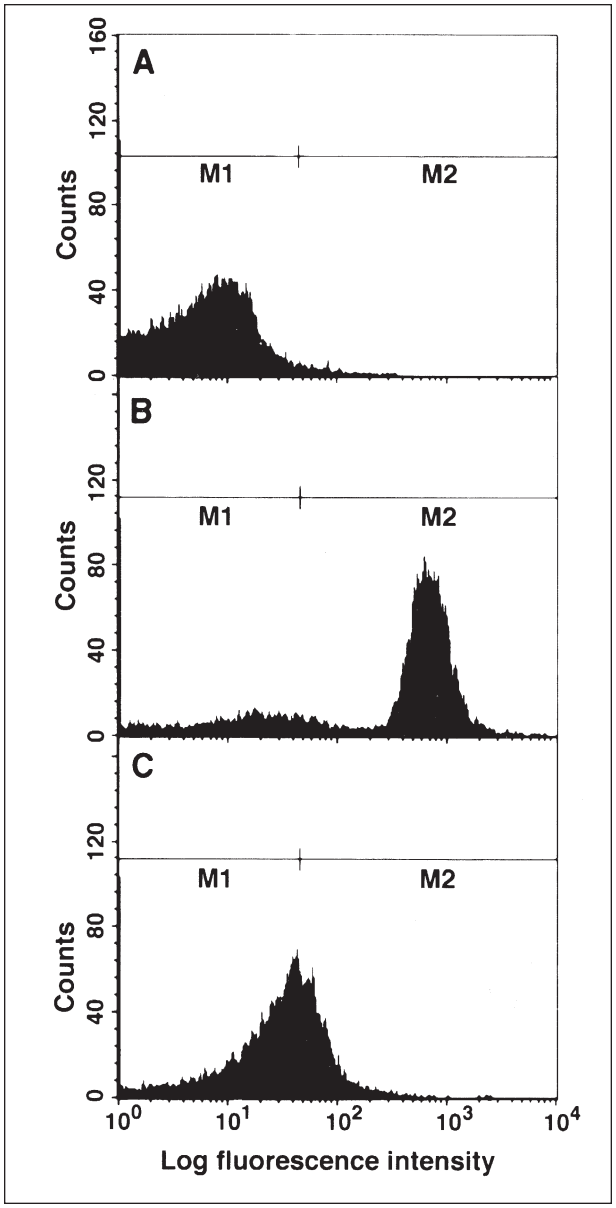

Figure 5—Histograms of flow cytometry of platelet-membrane GPIIIa in samples of platelets from an unaffected dog that were labeled with fluorescein isothiocyanate (FITC)-conjugated murine monoclonal antibody to *Aspergillus niger* glucose oxidase (isotype negative-control sample; A), platelets from an unaffected dog that were labeled with FITC-conjugated murine monoclonal antibody to human GP IIIa (CD61; positive-control sample; B); and thrombasthenic platelets from an affected Otterhound labeled with FITC-conjugated anti-human GP IIIa; C). The M1 region delineates low intensity platelet autofluorescence. The M2 region delineates the region of fluorescence signal associated with platelet binding of FITC-conjugated CD61 antibody to GPIIIa. Fluorescence intensity reflects the extent of labeling of the population of gated cells. Each histogram corresponds to 10,000 events. Data are from a single representative experiment.

Otterhound with an isotype FITC-conjugated mouse monoclonal antibody (IgG1) to *Aspergillus niger* glucose oxidase as a negative-control sample yielded background fluorescence intensity similar to the autofluorescence of unlabeled platelets (**Fig 5**). In contrast, platelets from a clinically normal dog that were labeled

	367									374		
	D	**L**	**D/H**	**R**	**D**	**G**	**Y**	**N**	**D**	**I/V**	**A**	**V**
Human	GAC	CTC	GAC	CGG	GAT	GGC	TAC	AAT	GAC	ATT	GCA	GTG
Dog	GAC	CTC	GAC	CGG	GAC	GGC	TAC	AAC	GAT	GTT	GCA	GTG
OH	GAC	CTC	GAC	CGG	GAC	GGC	TAC	AAC	GAT	GTT	GCA	GTG
OHC	GAC	CTC	G/C AC	CGG	GAC	GGC	TAC	AAC	GAT	GTT	GCA	GTG
Affected	GAC	CTC	CAC	CGG	GAC	GGC	TAC	AAC	GAT	GTT	GCA	GTG

Figure 6—Gene sequence that encodes the third calcium-binding domain of platelet GPIIb in humans, 4 clinically normal dogs representing various breeds, 2 unaffected Otterhounds (OH), 1 Otterhound that was a carrier for the trait (OHC), and 2 GT-affected Otterhounds (affected). Letter abbreviations for each encoded amino acid are listed above the gene codons. The GT-affected Otterhounds have a substitution at codon 367 as a result of a nucleotide change from G to C at position 1100, compared with clinically normal dogs and humans. Notice that the carrier Otterhound has both G and C at this location. A conservative substitution was detected at position 374 when comparing human and canine sequences. Notice that this area is highly conserved between human and canine sequences.

with FITC-conjugated mouse monoclonal antibody to human platelet GPIIIa had intense fluorescence in the population of gated cells. When labeled and probed in an identical manner, platelets from the affected Otterhound had only a slight rightward shift in fluorescence intensity, compared with results for the isotype control sample. Fluorescence intensity was 10- to 15-fold higher in platelets from the clinically normal dog, compared with those from the affected Otterhound.

Numbering systems for the cDNA sequences of the genes encoding platelet GPIIb and GPIIIa and the amino acids encoded by these genes have been variously reported as including or not including the leader sequence for the proteins. We used both systems in the study reported here. The cDNA sequence for the gene encoding GPIIIa in affected Otterhounds was identical to that described in clinically normal mixed-breed dogs (Genbank accession No. AF116270).[25] Two nucleotide changes resulting in amino acid substitutions were found in the gene encoding for GPIIb (Genbank accession, No AF153316).[15] One change was C to T at 1601 (C to T at 1508) in exon 17 resulting in substitution of a serine for a proline at amino acid position 534 (503). This change was observed in 2 affected Otterhounds but not in 4 other dogs of other breeds. The 2 unaffected Otterhounds and the carrier Otterhound were heterozygous at this position, because they had both C and T in their cDNA. The other change observed was G to C at 1193 (G to C at 1100) in exon 12 resulting in substitution of histidine for aspartic acid at amino acid position 398 (367) in the third calcium-binding domain (**Fig 6**). This change was observed in both affected Otterhounds but not in the 4 dogs of other breeds or in the unaffected Otterhounds. The carrier Otterhound was heterozygous at this position, because it had both G and C.

Discussion

Otterhounds with thrombasthenic thrombopathia that were identified in the late 1980s and early 1990s had platelets with functional and biochemical features similar to those described in humans with type-I GT.

Protein electrophoresis confirmed a lack of detectable platelet GPIIb and GPIIIa and normal quantities of GPIb and other major surface GP. Although ancestors of these dogs were believed to have a combined defect (GT and Bernard Soulier's disease), evidence of a combined defect could not be found. In the study reported here, existence of type-I GT in affected Otterhounds has now been verified at the molecular level.

The cDNA sequence for the gene encoding platelet GPIIb in Otterhounds with thrombasthenic thrombopathia had 2 changes resulting in amino acid substitutions. One change in exon 17 is probably a polymorphism. This polymorphism may be specific for Otterhounds, because other breeds of dogs (Great Pyrenees, Basset Hound, and mixed-breed dogs) did not have this nucleotide difference. The resulting substitution of a serine for a proline was interesting in light of the fact that platelet GPIIb in humans also has a serine in this position.[26] This suggests that the substitution does not impact the stability of the GPIIb-IIIa complex. This polymorphism is unsuitable as a marker for the defect, because 2 unaffected Otterhounds as well as a carrier Otterhound were heterozygous for this nucleotide change.

The amino acid substitution in exon 12 probably has profound importance. Substitution of histidine for aspartic acid within a highly conserved calcium-binding domain is likely to result in severe destabilization of the GPIIb-IIIa complex and lack of expression of the complex on the surface of platelets. Substitution of histidine in this location would result in a change in charge, but it also would introduce an amino acid with a bulky imidazole ring structure that would likely impact the overall structure and stability of the complex. This nucleotide change correlated well with disease expression, because 2 unaffected Otterhounds did not have this change, whereas a carrier Otterhound was heterozygous at this location. Supportive evidence that the defect involves the gene encoding for GPIIb was provided by the detection of small amounts of GPIIIa on the surface of platelets in affected Otterhounds. In humans with type-I GT that are the result of mutations in the gene encoding for GPIIb, results of flow cytometry revealed the existence of small quantities of GPIIIa.[12] Expression of vitronectin receptors is enhanced in these patients, and it is speculated that anti-GPIIIa antibodies detect this receptor during flow cytometry.[11,27] In contrast, platelets from humans with type-I GT that are the result of mutations in the gene encoding for GPIIIa do not have detectable binding of antibodies to GPIIIa during flow cytometry. Antibodies to canine platelet GPIIb were not available; therefore, GPIIb concentrations could not be evaluated by this method.

An identical molecular defect has not been described in humans with GT. However, changes in amino acids between calcium-binding domains of GPIIb have been described.[28-32] Ruan et al[28] proposed that amino acid substitutions between calcium-binding domains that result in a change in charge cause type-I GT, whereas amino acid substitutions that are charge-neutral are associated with type-II GT. The former types of defects result in inability of the GPIIb-IIIa complex to remain folded during transition from the endoplasmic reticulum to the Golgi apparatus, whereas the latter types of defects result in slowed processing and movement of the GPIIb-IIIa complex to the platelet surface. Type-I GT attributable to deletion of 2 amino acids in the fourth calcium-binding domain of GPIIb has been described in humans.[33] In that defect, heterodimer assembly of GPIIb-IIIa was not impaired, but further intracellular processing failed, which suggested that the calcium-binding domains are important for stability and transport of the complex to the cell surface. The authors of that report speculated that mutated sequences resulted in malfolded IIb proteins, and although this did not impair the ability of the protein to form a complex with IIIa, it did result in retention and degradation of the complex within the endoplasmic reticulum. This theory was supported in experiments of mutagenesis: when any of the 4 calcium-binding domains were deleted, the GPIIb-IIIa heterodimer still formed, but transport to the cell surface failed.

Although experiments involving site-directed mutagenesis of GPIIb and coupling with wild-type GPIIIa that result in expression of the mutation described here will be needed to verify that this mutation is the cause of type-I GT in thrombasthenic Otterhounds, it is likely that mechanisms resulting in GT in dogs will be similar or identical to those that cause GT in humans. Determining the molecular basis for GT in dogs provides an excellent opportunity to study structure-function relationships of this complex as well as providing potential models for gene therapy. This information also provides a mechanism for identifying Otterhounds that are carriers of GT that is superior to analysis of results of platelet aggregation, which often can be equivocal.

[a]Sepharose 4B, Pharmacia Fine Chemicals, Upsala, Sweden.
[b]Pentex fraction V, Miles Laboratories, Elkhart, Ind.
[c]Model 550-VS, Chronolog Corp, Havertown, Pa.
[d]Fibriquick (100 U/ml), Organon Teknika Corp, Durham, NC.
[e]Simplate II, Organon Teknika, Durham, NC.
[f]Whatman 1, Fisher Scientific Co, NJ.
[g]Vetbond, 3-M Co, St Paul, Minn.
[h]Model PT 10/35 homogenizer, Brinkmann Instruments, Westbury, NY.
[i]Gilford Model 2400, Dupont Instruments, Wilmington, Del.
[j]A6793, Sigma Chemical Co, St Louis, Mo.
[k]Coulter S+IV, Coulter Electronics, Hialeah, Fla.
[l]Clone Y2/51, Dako Corp, Carpinteria, Calif.
[m]IgG1 isotype control, Dako Corp, Carpinteria, Calif.
[n]FACSCalibur, Becton Dickinson, San Jose, Calif.
[o]Qiaquick gel extraction kit, Qiagen, Valencia, Calif.
[p]ABI Prism dye terminator cycle sequencing ready reaction kit with Ampli*Taq* DNA polymerase, Perkin-Elmer Corp, Norwalk, Conn.
[q]Applied Biosystems 373 A DNA sequencer, Perkin-Elmer Corp, Norwalk, Conn.

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