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# Platelet Function Defect in a Thoroughbred Filly

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A 2-year-old Thoroughbred filly presented to the Veterinary Medical Teaching Hospital (VMTH) at the University of California, Davis, for evaluation of a bleeding disorder. The bleeding tendency was 1st noted 3 weeks before presentation on the morning after the horse was pin fired. Prolonged bleeding was noted after intramuscular injection of procaine penicillin G, and the trainer noted blood from the horse's right nostril 2 days before presentation. The filly had been seen by a referral practice, which found a prolonged prothrombin time but no other problems. The horse received an injection of vitamin K intravenously the day before presentation. The current owners purchased the horse ~1 year before the initial bleeding episode, during which time she had not injured herself or had any intramuscular injections. The filly had received 1 dose PO of a dexamethasone (5 mg) and trichlormethiazide (200 mg) combination[a] and 1 or 2 doses of phenylbutazone (unknown amount) but had not received aspirin. The exact dates of administration of these drugs were not known, but they were not within the week before presentation. She had never bled from venipuncture sites.

On physical examination at the VMTH, the filly had serous fluid oozing from some pin firing sites. She had decreased gut sounds and several small oral abrasions. Results of initial hemostasis testing were as follows: 138 $\times$ $10^3$ platelets/$\mu$L (reference range 100–350 $\times$ $10^3$ platelets/$\mu$L); von Willebrand factor, 85% (reference range 70–80%); prothrombin time, 13.4 seconds (reference range 14–17 seconds); activated partial thromboplastin time, 53.4 seconds (reference range 31.5–48.5 seconds); factor VIII, 40% (reference range 50–200%); factor IX, 92% (reference range 50–150%); factor XI, 56% (reference range 60–150%); factor XII, 105% (reference range 60–150%); antithrombin, 108% (reference range 90–100%). On blood smear examination, platelets often had an elongated shape but were otherwise of unremarkable morphology. Mild abnormalities in coagulation tests were considered insufficient to explain the severity of clinical bleeding but possibly were indicative of low-grade consumption of coagulation factors. The filly's template bleeding time[b] was >120 minutes, whereas that of a control horse was 5.5 minutes. On the basis of these findings, it was determined that the most likely cause of the horse's bleeding diathesis was a defect in platelet function. Additional testing was performed to characterize the putative defect.

Optical aggregometry[c] of control horses and the filly was performed with platelet-rich plasma (PRP) or washed platelet preparations. Aggregation was tested with multiple agonists,[d] including adenosine diphosphate (ADP), collagen, collagen-related peptide, ristocetin, thrombin, and U46619. The horse's platelets had normal aggregation in response to ADP, the thromboxane $A_2$ ($TXA_2$) analog U46619, and ristocetin. There was mildly decreased aggregation in response to 0.5 U/mL thrombin but not to 1 U/mL thrombin.

The filly's platelets did not respond to collagen. Because of the striking lack of reactivity, aggregometry was performed with a synthetic agonist, collagen-related peptide (CRP), that selectively binds to the glycoprotein VI (GPVI) collagen receptor.[1,2,3] The filly's platelets had diminished aggregation to CRP (1 $\mu$g/mL) and delayed but complete aggregation at 5 $\mu$g/mL. Collagen-related peptide studies were conducted with washed platelets[4] in a buffer containing 2 mM $CaCl_2$, suggesting that the addition of calcium might affect aggregation in citrate-anticoagulated PRP as well. This was in fact the case: calcium-treated PRP (2 mM $CaCl_2$) stimulated with 10 $\mu$g/mL collagen resulted in marked aggregation, whereas previous experiments without the addition of calcium had no response. The filly's platelets also showed aggregation in response to collagen with PRP made from blood collected into sodium heparin, an anticoagulant that is not a calcium-chelating agent.

Standard clot retraction assays[5] performed on whole blood and PRP did not show differences between the filly and 3 control samples (data not shown).

Platelet adhesion to collagen was evaluated with the use of microtiter wells coated with Type I collagen.[e] The wells were 1st incubated with a dilution series (1.8 $\times$ $10^8$ to 1.8 $\times$ $10^5$ platelets/$\mu$L) of platelets washed and suspended in Tyrodes-Hepes buffer containing 1 mM $MgCl_2$ then washed. Adhered platelets were detected with a vital dye converted by mitochondria to a product that absorbs light at 570 nm.[f] The platelets of the horse and a control had similar adhesion (data not shown).

Platelet ultrastructural morphology was examined by electron microscopy. The horse's platelets had an elongated shape, consistent with the morphology noted on blood smear examination, and contained both alpha and dense granules (Fig 1).

Western blot assays[g] were performed for a number of platelet proteins, and bound antibodies[h] were detected by chemiluminescence.[i] Assays for a number of proteins, including both chains of the $\alpha_{IIb}\beta_{3a}$ (GPIIa-IIIb) integrin and the glycoproteins GPIb and GPVI, demonstrated that these proteins were present in both horse and control platelets (data not shown). Polyacrylamide gel electrophoresis of

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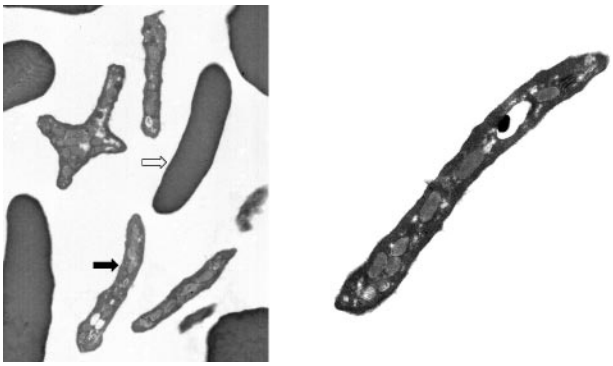

**Fig 1.** Low-magnification (left) electron micrograph of platelets (black arrow) and erythrocytes (open arrow) and high-magnification (right) electron micrograph of a platelet. Note numerous alpha granules and a single dense granule in the platelet shown in the high-magnification image.

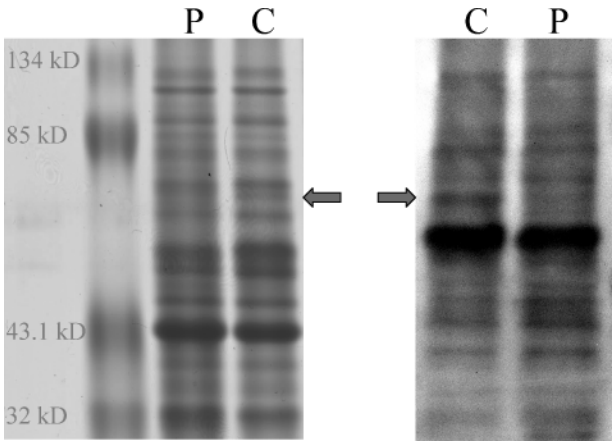


**Fig 2.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis–stained gel (left) and western blot for phosphotyrosine (right), showing a missing or markedly decreased band of ~70–79 kd (arrows). P, patient; C, control.

whole platelet lysate preparations showed that the horse's platelets had a missing or markedly decreased band in the range of 70–79 kd, and western blotting for phosphotyrosine (PY) showed a missing or markedly decreased band of similar mass (Fig 2). However, western blot analysis for intracellular signaling molecules critically involved in GPVI signaling—Syk, fyn, lyn, and SLP-76—did not reveal a difference in expression between the presenting horse and a control (data not shown).

A presumptive diagnosis of a platelet function defect in this horse was made on the basis of the normal platelet concentration, coagulation test abnormalities considered too mild to explain the clinical bleeding, and a markedly prolonged template bleeding time. This diagnosis was confirmed by the finding of severely impaired platelet aggregation in response to collagen. Collagen aggregation experiments were repeated on multiple occasions several months after the horse presented to the VMTH, during which time she did not receive any medications, with similar results. The horse did not have any clinical or clinicopathologic evidence of systemic disease, and results of Coggins testing for equine infectious anemia virus were negative. The horse had additional bleeding episodes in the months after presentation, including profuse oral bleeding with eruption of permanent teeth and an episode of prolonged epistaxis. On the basis of these findings, it was considered likely that the platelet defect was congenital, rather than acquired.

A clinical bleeding tendency and abnormal platelet response to collagen in vitro are features of Chediak Higashi syndrome (CHS), a condition previously recognized in people and many veterinary species, but not horses.[6] However, this horse lacked other classic features of CHS, such as recurrent pyogenic infections, dilute hair coat color, and prominent cytoplasmic inclusions in blood cells.[6,7] Moreover, electron microscopy demonstrated the presence of dense granules, which are absent or markedly decreased in cases of CHS.[6,7] The elongated shape of the horse's platelets suggests that they have structural, as well as functional, abnormalities. However, 2 of the authors (MMF, FT) have noted similar platelets from horses without any bleeding tendencies.

Other inherited platelet function defects described in humans and veterinary species include Glanzmann's thrombasthenia and Bernard-Soulier syndrome, diseases characterized by quantitative or qualitative deficiencies in the major platelet surface receptors $\alpha_{IIb}\beta_{3a}$ (fibrinogen receptor) and GPIb (vWF receptor), respectively. Glanzmann's thrombasthenia is characterized by poor aggregation in response to most agonists and by severely impaired clot retraction,[8] whereas the horse in this report responded normally to most agonists and had normal clot retraction. Bernard-Soulier syndrome platelets have a defect in the GPIb-V-IX complex and show normal clot retraction along with normal responses to ADP and collagen, but absent responses to ristocetin.[9] This horse's platelets express proteins recognized by antibodies directed against both chains of $\alpha_{IIb}\beta_{3a}$ and against GPIb.

Platelet-collagen interactions involve multiple receptors and signal transduction molecules.[10,11] It is generally accepted that the $\alpha_2\beta_1$ (GPIa-IIa) integrin and the GPVI glycoprotein are the main receptors involved in adhesion to and activation in response to collagen, respectively.[3,10] Glycoprotein VI is coexpressed with an immunoglobulin receptor, FcR$\gamma$, that has an immunoreceptor tyrosine-linked activation motif.[12,13] Binding of collagen to GPVI initiates a phosphorylation cascade involving Src family tyrosine kinases, including Syk, lyn, fyn, and adapter proteins such as SLP-76 and Lat; downstream events include activation of phospholipases, including PLC$\gamma_2$ and cPLA$_2$; mobilization of intracellular calcium; and eventual activation of the arachidonic acid pathway.[3,10,14,15] There is conflicting evidence about whether platelet activation by collagen requires secretion of the arachidonic acid metabolite TXA$_2$.[2,16,17] Some evidence indicates that TXA$_2$ plays a lesser role in the activation of platelets in horses than in people.[18] Thromboxane A$_2$ has a role in feedback activation, but is not required for irreversible aggregation, of equine platelets in response to collagen.[18] Other differences between equine

and human platelets (eg, in response to ADP) could be from differences in intracellular signaling pathways.[19]

These studies indicate that the filly's bleeding tendency is unlikely to be attributable to defective expression of either $\alpha_2\beta_1$ or GPVI. Adhesion assay results provide functional evidence of normal $\alpha_2\beta_1$ expression, and western blot and aggregometry results provide immunologic and functional evidence, respectively, of GPVI expression.

The horse's response to both CRP and U46619 supports the hypothesis that her platelet defect is downstream of the GPVI collagen receptor and upstream of the $TXA_2$ receptor. Together with the finding that her severely impaired in vitro platelet aggregation in response to collagen is obviated by the addition of physiologic concentrations of calcium, the results suggest a lesion involving defective intracellular signaling. Entry of $Ca^{2+}$ into the cell and subsequent tyrosine phosphorylation have been shown to be diminished in lymphocytes from a human with Scott syndrome, an inherited disorder of platelet procoagulant activity involving impaired exposure of phosphatidylserine on the outer leaflet of the plasma membrane.[20] However, the horse in this report does not fit the typical Scott syndrome profile characterized by a normal bleeding time and no demonstrable platelet aggregation abnormalities.[21] There is no direct evidence of a primary platelet receptor defect in this horse. However, the experimental results raise the possibility of impaired receptor function from defective ''inside-out'' signaling. When a platelet is activated, $\alpha_{IIb}\beta_{3a}$ undergoes a conformational change that allows it to bind fibrinogen and form the stable platelet-platelet interactions critical to normal hemostasis. In addition, other investigators have shown that blocking collagen binding to $\alpha_2\beta_1$, GPVI (receptors whose signaling pathways involve an increase in intracellular calcium concentration), or both impairs activation of $\alpha_{IIb}\beta_{3a}$.[22] It is thus reasonable to assume that a signaling defect downstream from collagen binding might result in impaired fibrinogen binding and a clinical presentation similar to Glanzmann's thrombasthenia.

There have been reports of knockout mice, lacking key signal transduction proteins involved in platelet collagen signaling, with platelet phenotypes similar to that of the filly in this report. For example, fyn −/− and lyn −/− knockout mice have impaired aggregation in response to CRP,[16] and SLP-76 −/− mice have impaired aggregation to collagen.[23] Although western blot assays for fyn, lyn, Syk, and SLP-76 do not show any of these proteins to be missing from the horse's platelets, the decreased expression of a band in the range of 70–79 kd that has positive staining for phosphotyrosine suggests that there might yet be a missing, underexpressed, or altered signal transduction protein. The specific platelet function defect has yet to be identified.

## Footnotes

[a] Naquasone, Schering-Plough, Kenilworth, NJ

[b] Simplate II R, Organon Teknika, Durham, NC

[c] Chronolog 490D, Chrono-log, Haverton, PA

[d] Agonists used for aggregometry: ADP (Chrono-log, Haverton, PA), collagen (Chrono-log), CRP (kindly provided by Dr Steve P. Watson, University of Oxford, UK), ristocetin (Chrono-log), thrombin (Sigma, St Louis, MO), U46619 (Calbiochem, San Diego, CA)

[e] BioCoat Collagen I–coated 96-well plates, Becton Dickinson, Bedford, MA

[f] Promega CellTiter 96, nonradioactive cell proliferation assay, Madison, WI

[g] Bio-Rad Laboratories, Hercules, CA

[h] Primary antibodies used for western blotting: $\alpha_{IIb}$ (monoclonal antibody [mAb], Immunotech, Marseilles, France), $\beta_{3a}$ (mAb, DAKO, Carpenteria, CA), fyn (polyclonal antibody [pAb], Santa Cruz Biotechnology, Santa Cruz, CA), GPIb (mAb, kindly provided by Dr Dermot Kenney, University of Dublin, Ireland), GPVI (pAb, kindly provided by Dr Kenneth J. Clemetson, Theodor Kother Institute, Berne, Switzerland), lyn (mAb, Santa Cruz Biotechnology), PY (mAb, Transduction Laboratories, Lexington, KY), Src (mAb, Upstate Biotechnology, Lake Placid, NY), Syk (mAb, Santa Cruz Biotechnology), SLP-76 (mAb, Santa Cruz Biotechnology). Secondary antibodies: goat anti-mouse IgG-AP (pAB, Pierce, Rockford, IL), goat anti-rabbit IgG-AP (pAB, Pierce), goat anti-rabbit IgG-HRP (Bio-Rad Laboratories, Hercules, CA)

[i] CDP-Star, Roche Molecular Biochemicals, Indianapolis, IN

## References

1. Knight CG, Morton LF, Onley DJ, et al. Collagen-platelet interaction: Gly-Pro-Hyp is uniquely specific for platelet GpVI and mediates platelet activation by collagen. Cardiovasc Res 1999;41:450–457.

2. Quinton TM, Ozdener F, Dangelmaier C, et al. Glycoprotein VI–mediated platelet fibrinogen receptor activation occurs through calcium-sensitive and PKC-sensitive pathways without a requirement for secreted ADP. Blood 2002;99:3228–3234.

3. Nieswandt B, Watson SP. Platelet-collagen interaction: Is GPVI the central receptor? Blood 2003;102:449–461.

4. Polgar J, Clemetson JM, Kehrel BE, et al. Platelet activation and signal transduction by convulxin, a C-type lectin from *Crotalus durissus terrificus* (tropical rattlesnake) venom via the p62/GPVI collagen receptor. J Biol Chem 1997;272:13576–13583.

5. Wilcox DA, Paddock CM, Lyman S, et al. Glanzmann thrombasthenia resulting from a single amino acid substitution between the second and third calcium-binding domains of GPIIb. Role of the GPIIb amino terminus in integrin subunit association. J Clin Invest 1995;95:1553–1560.

6. Meyers KM. Chediak-Higashi syndrome. In: Feldman BF, Zinkl JG, Jain JG, eds. Schalm's Veterinary Hematology, 5th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2000:971–975.

7. Shiraishi M, Ogawa H, Ikeda M, et al. Platelet dysfunction in Chediak-Higashi syndrome–affected cattle. J Vet Med Sci 2002;64:751–760.

8. Boudreaux MK, Lipscomb DL. Clinical, biochemical, and molecular aspects of Glanzmann's thrombasthenia in humans and dogs. Vet Pathol 2001;38:249–260.

9. Lopez JA, Andrews RK, Afshar-Kharghan V, Berndt MC. Bernard-Soulier syndrome. Blood 1998;91:4397–4418.

10. Watson SP. Collagen receptor signaling in platelets and megakaryocytes. Thromb Haemost 1999;82:365–376.

11. Cho MJ, Liu J, Pestina TI, et al. The roles of alpha IIb beta 3-mediated outside-in signal transduction, thromboxane A2, and adenosine diphosphate in collagen-induced platelet aggregation. Blood 2003;101:2646–2651.

12. Clemetson JM, Polgar J, Magnenat E, et al. The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcαR and the natural killer receptors. J Biol Chem 1999;274:29019–29024.

13. Nieswandt B, Bergmeier W, Schulte V, et al. Expression and function of the mouse collagen receptor glycoprotein VI is strictly

dependent on its association with the FcRγ chain. J Biol Chem 2000; 275:23998–24002.

14. Leo L, Di Paola J, Judd BA, et al. Role of the adapter protein SLP-76 in GPVI-dependent platelet procoagulant responses to collagen. Blood 2002;100:2839–2844.

15. Quek LS, Pasquet JM, Hers I, et al. Fyn and Lyn phosphorylate the Fc receptor γ chain downstream of glycoprotein VI in murine platelets, and Lyn regulates a novel feedback pathway. Blood 2000; 96:4246–4253.

16. Rink TJ, Sage SO. Calcium signaling in human platelets. Annu Rev Physiol 1990;52:431–449.

17. Nakamura T, Jamieson GA, Okuma M, et al. Platelet adhesion to native type I collagen fibrils. Role of GPVI in divalent cation-dependent and -independent adhesion and thromboxane A2 generation. J Biol Chem 1998;273:4338–4344.

18. Rickards KJ, Andrews MJ, Waterworth TH, et al. Differential effects of phosphodiesterase inhibitors on platelet activating factor (PAF)- and adenosine diphosphate (ADP)-induced equine platelet aggregation. J Vet Pharmacol Ther 2003;26:277–282.

19. Heath MF, Evans RJ, Poole AW, et al. The effects of aspirin and paracetamol on the aggregation of equine blood platelets. J Vet Pharmacol Ther 1994;17:374–378.

20. Martinez MC, Martin S, Toti F, et al. Significance of capacitative Ca2+ entry in the regulation of phosphatidylserine expression at the surface of stimulated cells. Biochemistry 1999;38:10092–10098.

21. Zwaal RF, Comfurius P, Bevers EM. Scott syndrome, a bleeding disorder caused by defective scrambling of membrane phospholipids. Biochim Biophys Acta 2004;1636:119–128.

22. Nakamura T, Kambayashi J, Okuma M, Tandon NN. Activation of the GP IIb-IIIa complex induced by platelet adhesion to collagen is mediated by both alpha2beta1 integrin and GP VI. J Biol Chem 1999;274:11897–11903.

23. Clements JL, Lee JR, Gross B, et al. Fetal hemorrhage and platelet dysfunction in SLP-76–deficient mice. J Clin Invest 1999;103: 19–25.