

LETTERS TO THE EDITOR

Scott syndrome dogs have impaired coated-platelet formation and calcein-release but normal mitochondrial depolarization

M. B. BROOKS,* J. L. CATALFAMO,* P. FRIESE† and G. L. DALE†

*Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY; and †Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

To cite this article: Brooks MB, Catalfamo JL, Friese P, Dale GL. Scott syndrome dogs have impaired coated-platelet formation and calcein-release but normal mitochondrial depolarization. *J Thromb Haemost* 2007; 5: 1972–4.

Dual agonist stimulation of platelets with thrombin plus collagen, or thrombin plus convulxin (a ligand of the collagen receptor glycoprotein VI), generates a subpopulation of highly procoagulant cells known as ‘coated’ platelets [1]. Coated-platelets represent approximately 30% of the total platelet population and display a combination of characteristic features including expression of phosphatidylserine (PS), surface retention of alpha granule proteins, permeability to calcein, and release of membrane-derived microparticles [2–6]. Furthermore, recent studies demonstrate that loss of mitochondrial membrane potential via formation of a mitochondrial permeability transition pore (MPTP), a key feature of the intrinsic apoptotic pathway, is also an integral event leading to platelet membrane PS externalization and coated platelet formation [6]. While *in vitro* experimental systems indicate that the proportion of coated-platelets correlates with thrombin generation [3,5], the role of coated-platelets in physiologic and pathologic thrombus formation *in vivo* is still under investigation.

In a series of experiments, we investigated the formation of coated-platelets utilizing a canine model of Scott syndrome. This trait was identified in a colony of German shepherd dogs (GSD) with an autosomal recessive bleeding diathesis characterized by a specific deficiency of platelet procoagulant activity [7]. Affected GSD demonstrate the pathognomonic platelet phenotype of Scott syndrome (i.e. a lack of PS expression and failure of membrane microvesiculation upon activation with calcium ionophore) [8,9]. Furthermore, Scott GSD platelets do not generate prothrombinase activity in response to ionophore or the physiologic agonists thrombin and collagen [7]. We hypothesized that dual agonist stimulation of Scott GSD platelets would not only fail to elicit PS externalization, but that Scott GSD platelets would be incapable of displaying the characteristic features of coated-platelets [1].

Coated-platelets were produced by thrombin plus convulxin stimulation of platelet-rich plasma (PRP) prepared from healthy control dogs and clinically affected GSD. All activation experiments were performed in a 100 μ L (total) assay volume containing 1 μ L PRP and the following reagents (final concentrations): 1 mg mL⁻¹ BSA, 2 mM CaCl₂, 1 mM MgCl₂, 1 μ g mL⁻¹ biotin-fibrinogen, 500 ng mL⁻¹ convulxin, 0.5 U mL⁻¹ bovine thrombin, 0.4 mM gly-pro-arg-pro-NH₂, 150 mM NaCl, and 10 mM HEPES, pH 7.5. The reaction tubes were incubated at 37° for 10 min, and individually labeled, as previously described [4,6,7,10], with the following specific markers of coated-platelets: Annexin V-fluorescein isothiocyanate (FITC) to detect PS expression; phycoerythrin-streptavidin and FITC-abciximab to label surface bound biotinylated fibrinogen and identify platelets; calcein-AM to detect calcein release; and JC-1 to monitor the loss of mitochondrial membrane potential, denoting formation of the MPTP.

Scott GSD platelets failed to bind fibrinogen, externalize PS or release calcein upon dual agonist stimulation, in contrast to

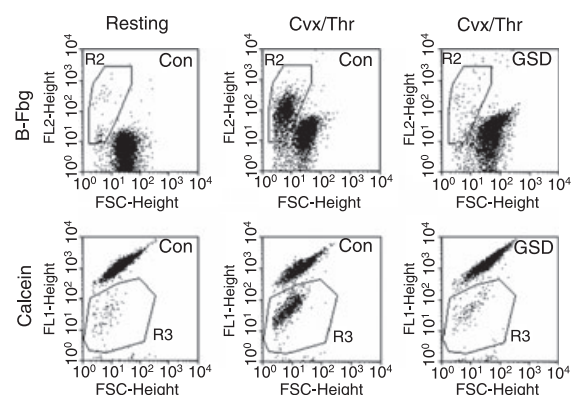


Fig. 1. Flow cytometric analysis of control and Scott German shepherd dogs (GSD) platelets. Platelets were activated with convulxin plus thrombin (Cvx/Thr), and binding of biotin-fibrinogen (B-Fbg; top row) or calcein-release (bottom row) was monitored. Con indicates platelets from control dogs, and GSD indicates platelets from Scott GSD. In the top row, activation of control dog platelets induces a new population of cells with bound fibrinogen (R2); this population is lacking in stimulated GSD platelets. Similarly, the bottom row indicates that control platelets upon activation release calcein (R3) while GSD platelets do not.

Correspondence: Marjory B. Brooks, Comparative Coagulation Section, Animal Health Diagnostic Center, Cornell University, Upper Tower Road, Ithaca, NY 14852-5786, USA.

Tel.: +1 607 275 0622; fax: +1 607 275 0720; e-mail: mbb9@cornell.edu

Received 23 February 2007, accepted 26 June 2007

Table 1 Parameters associated with coated-platelet formation in Scott German shepherd dogs (GSD) and control dogs

Subjects	n	Flow cytometry markers			
		Fibrinogen*	Annexin [†]	Calcein [‡]	JC-1 [§]
		% Positive cells		% Negative cells	
Control dogs	5	37.3 ± 6.1	53.9 ± 10.4	36.6 ± 5.6	60.6 ± 10.1
Scott GSD	5	5.1 ± 2.2**	4.2 ± 3.9**	3.3 ± 0.9**	65.3 ± 8.6

Control dogs included one GSD, one GSD/labrador mix (related to Scott GSD), two hounds, and one terrier.

Data represent mean ± 1 SD.

*Surface-bound, biotinylated fibrinogen.

[†]Fluorescein isothiocyanate-Annexin V, denoting membrane expression of phosphatidylserine.

[‡]Loss of calcein fluorescence, indicating calcein-release.

[§]Loss of JC-1 fluorescence (FL1), denoting mitochondrial permeability transition pore formation.

**Indicates that means are significantly different from control dog values ($P \leq 0.006$).

the responses observed for control platelets (Fig. 1 and Table 1). However, there were no significant differences between Scott GSD and control dogs in the percentages of stimulated platelets demonstrating a shift in JC-1 fluorescence (Table 1). These data demonstrate that the platelet phenotype of canine Scott syndrome comprises a failure to generate coated-platelets in response to dual agonist stimulation, in spite of an apparently normal formation of MPTP. Previously we have defined coated-platelets by the retention of surface-bound fibrinogen [6] with the recognition that other intermediate markers of coated-platelet formation (i.e. MPTP, calcein release and PS exposure) are required events but not sufficient to produce coated-platelets [6].

Our previous studies of Scott GSD revealed normal aggregation, secretion, and clot retraction in response to thrombin and/or collagen stimulation [7], indicating that the receptors and signaling pathways for these agonists remain intact and that the glycoprotein IIb/IIIa complex on these platelets is functional. In the current study, we observed a complex pattern of abnormalities in markers of coated-platelets (Table 1). As pedigree studies of the Scott GSD colony indicate a simple autosomal recessive trait [7], it is likely that all these anomalies are caused by a single gene defect. One possible explanation for this finding is that a pathway of sequential events is required for coated-platelet formation and that interruption of any upstream event abolishes all downstream events. In this model, the combined stimulation of thrombin and collagen receptors induces a loss of mitochondrial membrane potential in only a subset of platelets. The formation of MPTP in this subset of cells results in the release of cytoplasmic calcein, externalization of PS and production of coated-platelets as indicated by fibrinogen retention. According to this model, the Scott GSD platelet defect is downstream of MPTP formation, but upstream of calcein release, PS exposure and fibrinogen retention. In support of this proposal, pharmacologic inhibition of MPTP formation [6] has been shown to prevent calcein loss, PS externalization and coated-platelet formation. Similarly, genetic disruption of MPTP formation [11] results in a failure of PS exposure and coated-platelet formation; calcein-release in this system has yet to be measured.

Certain phenotypic features of Scott GSD differ from human Scott syndrome patients. In contrast to human Scott cells [9,12], Scott GSD have no demonstrable abnormality in red cell PS externalization [7] or in platelet expression of the lipid transporter, ABCA-1 [13]. The hallmark of Scott syndrome, an inability to express PS on activated platelets, may therefore result from more than a single molecular defect. Nevertheless, Scott GSD platelets provide a unique model system to further elucidate the pathways leading to stimulated PS externalization and the role of platelet membrane PS in localization and amplification of thrombin generation.

Contributions

M. B. Brooks designed and performed research, analyzed data and wrote manuscript. J. L. Catalfamo assisted in research design, analyzed data and wrote manuscript. P. Friese provided critical reagents, analyzed data and standardized assay conditions. G. L. Dale designed research, analyzed data and wrote manuscript.

Acknowledgements

This work was supported by the National Institutes of Health (GLD) and research funds from the Comparative Coagulation Section, Animal Health Diagnostic Center, Cornell University.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

- 1 Dale GL. Coated-platelets: an emerging component of the procoagulant response. *J Thromb Haemost* 2005; 3: 2185–92.
- 2 Heemskerk JW, Vuist WM, Feijge MA, Reutelingsperger CP, Lindhout T. Collagen but not fibrinogen surfaces induce bleb formation, exposure of phosphatidylserine, and procoagulant activity of adherent platelets: evidence for regulation by protein tyrosine kinase-dependent Ca²⁺ responses. *Blood* 1997; 90: 2615–25.

- 3 Kempton CL, Hoffman M, Roberts HR, Monroe DM. Platelet heterogeneity: variation in coagulation complexes on platelet subpopulations. *Arterioscler Thromb Vasc Biol* 2005; **25**: 861–6.
- 4 Alberio L, Safa O, Clemetson KJ, Esmon CT, Dale GL. Surface expression and functional characterization of alpha-granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin. *Blood* 2000; **95**: 1694–702.
- 5 Kjalke M, Kjellv S, Rojkaer R. Preferential localization of recombinant factor VIIa to platelets activated with a combination of thrombin and a glycoprotein VI receptor agonist. *J Thromb Haemost* 2007; **5**: 774–80.
- 6 Remenyi G, Szasz R, Friese P, Dale GL. Role of mitochondrial permeability transition pore in coated-platelet formation. *Arterioscler Thromb Vasc Biol* 2005; **25**: 467–71.
- 7 Brooks MB, Catalfamo JL, Brown HA, Ivanova P, Lovaglio J. A hereditary bleeding disorder of dogs caused by a lack of platelet procoagulant activity. *Blood* 2002; **99**: 2434–41.
- 8 Weiss HJ. Scott syndrome: a disorder of platelet coagulant activity. *Semin Hematol* 1994; **31**: 312–9.
- 9 Zwaal RF, Comfurius P, Bevers EM. Scott syndrome, a bleeding disorder caused by defective scrambling of membrane phospholipids. *Biochim Biophys Acta* 2004; **1636**: 119–28.
- 10 Dale GL, Friese P, Batar P, Hamilton SF, Reed GL, Jackson KW, Clemetson KJ, Alberio L. Stimulated platelets use serotonin to enhance their retention of procoagulant proteins on the cell surface. *Nature* 2002; **415**: 175–9.
- 11 Jobe SM, Wilson KM, Leo L, Molkenin JD, Lentz SR, DiPaola J. Critical role for cyclophilin D and mitochondrial permeability transition pore (MPTP) in platelet activation. *Blood* 2006; **108**: 435a (Abstract).
- 12 Albrecht C, McVey JH, Elliott JI, Sardini A, Kasza I, Mumford AD, Naoumova RP, Tuddenham EG, Szabo K, Higgins CF. A novel missense mutation in ABCA1 results in altered protein trafficking and reduced phosphatidylserine translocation in a patient with Scott syndrome. *Blood* 2005; **106**: 542–9.
- 13 Catalfamo JL, Brooks MB, Ammersbach M, deMaster A. Platelet ABCA1 expression is normal in Scott syndrome dogs. *Blood* 2006; **108**: 57b (Abstract).

The cost-effectiveness of warfarin pharmacogenomics

D. L. VEENSTRA

Department of Pharmacy, University of Washington, Seattle, WA, USA

To cite this article: Veenstra DL. The cost-effectiveness of warfarin pharmacogenomics. *J Thromb Haemost* 2007; **5**: 1974–5.

Recently, the AEI-Brooking Joint Center for Regulatory Studies released a report via their website on the cost-effectiveness of warfarin pharmacogenomics [1]. The report estimated that testing for variants in the *CYP2C9* and *VKORC1* genes to guide initial dosing of warfarin therapy could provide \$1 100 000 000 in cost savings to the US healthcare system annually. The report also estimated that 85 000 serious bleeding events and 17 000 strokes could be avoided annually. These are overly optimistic projections.

Pharmacogenomics promises to be one of the first widespread applications of the genomics era to human health, and warfarin has leapt to the forefront as one of the best potential applications of pharmacogenomics for a commonly prescribed drug. Thus, warfarin pharmacogenomics has become an important case-study for genomic technologies, and there has been much excitement surrounding it. However, as with all healthcare technologies, care must be taken in its evaluation.

Correspondence: D. L. Veenstra, Department of Pharmacy, Pharmaceutical Outcomes Research and Policy Program, H-375 Health Sciences Center, PO Box 357630, Seattle, WA 98195-7630, USA.

Tel.: +1 206 616 1383; fax: +1 206 543 3835; e-mail: veenstra@u.washington.edu

Received 26 April 2007, accepted 28 June 2007

Given the importance of the topic, it is particularly disappointing that the AEI study contains major limitations that call into question the validity of its findings. Firstly, to date, there have not been any published clinical studies showing that warfarin pharmacogenomic testing improves patient outcomes. A technology cannot be cost-effective if it is not effective. Secondly, despite the lack of data on effectiveness, the report assumes that testing would essentially be 100% effective – lowering the bleeding risk in variant patients to that of wild types. Thirdly, the baseline risk of major bleeding used in the study, approximately 17.5% for the overall population, is two to four times higher than has been observed in prospective studies, and even in our study, from which it was derived [2]. It appears that the authors have applied cumulative incidences as annual rates. Fourth, the authors assume a reduction in the risk of stroke related to *VKORC1* genotyping, although no such trends have been observed clinically. Finally, the ranges explored in the probabilistic sensitivity analysis are not reflective of the uncertainty in the data.

There are other factors that were not assessed that may provide economic benefits, such as a reduction in hospital length of stay for inpatients, and reduced use of low-molecular-weight heparin resulting from patients reaching therapeutic International Normalized Ratio more quickly. A more careful evaluation of the economics of warfarin pharmacogenomics will require a basis in clinical trial data.