Comparative ultrastructural analyses of mouse, rabbit, and human platelets and fibrin networks

E. Pretorius,[1]* P. Humphries,[1,2] O.E. Ekpo,[1] E. Smit,[1] And C.F. Van Der Merwe[3]

[1]Department of Anatomy, School of Health Sciences, Faculty of Health Sciences, University of Pretoria, South Africa
[2]Department of Anatomy, University of Limpopo, Medunsa Campus, Garankuwa, South Africa
[3]Laboratory for Microscopy and Microanalysis, University of Pretoria, South Africa

Abstract

Platelets and fibrin play an important role in the coagulation process, where they are involved in the maintenance of hemostasis. Fibrin dysfunction is associated with the development of vascular complications, while proneness to the formation of tight and rigid fibrin networks is independently associated with thrombotic disease. Here we investigate the ultrastructure of human, rabbit, and mouse platelets and fibrin networks, using the scanning electron microscope. Human and rabbit fibrin and platelets, with regards to morphology as well as size of major and minor fibers compare well with each other. However, mouse fibers are much thinner and form a flimsy branching network. Platelet aggregate morphology of all three species compare well with each other. We conclude that rabbit platelet and fibrin networks could be used successfully when studying the effect of pharmaceutical products in preclinical trials, when looking at the effects of these products on morphology and ultrastructure.

Introduction

Platelets and fibrin play an important role in the coagulation process where they are involved in the maintenance of hemostasis as well as the initiation of repair following tissue injury (Herd and Page, 1994). Platelets are an important source of growth-promoting factors and in vivo their activation requires the initiation of a blood coagulation cascade when subendothe-lial tissue factor is exposed to the blood flow following either damage or activation of the endothelium (Butenas and Mann, 2002; Carmeliet and Collen, 1988; McVey, 1999; Nemerson, 1988) (Chart 1). However, fibrin dysfunction is associated with the development of vascular complications, while proneness to the formation of tight and rigid fibrin networks is independently associated with thrombotic disease. There are also genetic disease complexes that cause inborn errors of fibrinogen structure and are by definition congenital dysfibrinogenaemias. This anomaly results in a disturbance of the interchain disulphide bond, an ultra-structural defect, which interferes with fibrin polymerization (Pretorius et al., 2006). Furthermore, if platelets and fibrin function is disturbed by medication, it may lead to either bleeding or clotting in patients.

Morphology of both platelets, as well as fibrin networks may play an important role by providing additional information regarding dysfunctional hemostasis or in clinical trials where patients are exposed to different pharmaceuticals. Also, even in the preclinical trial period, where products are still tested on animal models like the mouse or rabbit model, ultrastructure of platelets and fibrin may be a valuable diagnostic tool. However, the analytic value of the morphology and ultrastructure of platelets and fibrin networks are often underestimated.

Although both rabbits and mice have been used successfully as animal models to study coagulopathies, the question that arises is whether there are indeed similarities between platelet activation via thrombin resulting in fibrin network formation in humans, rabbits, and mice. During the

process of coagulation, thrombin generation followed by fibrin clot formation takes place when tissue factor binds to a serine protease, factor VIIa (in blood). VIIa complex activates zym-ogen-factor IX and factor X. This generates thrombin that activates platelets and via a cascade process cleaves fibrinogen and factor XIII (Chart 1). Studies that confirm homologies between the coagulation cascade of humans, rabbits, and mice include research by James et al. (1997), Marx et al. (2000), Ruiz et al. (2000), and Humphries et al. (Comparative analysis of certain coagulation factors between human and rabbit blood samples, submitted for publication).

James et al. (1997) showed that intact and reduced patterns of mouse factor X in SDS-PAGE were similar to those of human factor X, and that mouse brain thromboplastin satisfactorily replaced rabbit brain thromboplastin in extrinsic activation of factor X in mouse plasma. The authors further suggest that their results are useful in making the mouse suitable for study of the mammalian blood coagulation pathways. Ruiz et al. (2000) found that homogeneous rabbit recombinant factor VII was fully active biologically as determined by prothrombin time assay in factor FVII-depleted plasmas, of both human and rabbit origin, using either human or rabbit thromboplastin.

According to Marx et al. (2000), based on in vitro studies, thrombin-activatable fibrinolysis inhibitor (TAFI) has been hypothesized as a link between coagulation and fibrinolysis. The authors also found that the deduced amino acid sequence of murine TAFI is highly identical to human TAFI.

Humphries et al., (submitted for publication) compared the levels of human and rabbit coagulation factors, with specific interest in factors II, V, VII, VIII, IX, X, and fibrinogen. The prothrombin time (measures how long blood takes to form a clot) and activated partial thromboplastin time was also measured. All experiments were performed on the Start 4 of Diagnos-tica Stago (Paris, France). All procedures were done according to the standardized operating procedures for determining human values and assay kits used were those typically used in human studies. Results indicated that rabbit factors are comparable to that of humans and therefore it is suggested that the intrinsic and extrinsic pathways of humans and rabbits are therefore comparable.

The question that also arises is whether there are differences between human, mouse and rabbit throm-bin. Human thrombin belongs to the trypsin family of serine proteases (Bouma and Mosnier, 2003) this is also true for mouse and rabbit thrombin. Human thrombin is a two chain enzyme composed of an NH_2-terminal A-chain (M_r = 6,000) and a COOH-terminal B-chain (M_r = 31,000), which remain covalently associated through a single disulfide bond. All three types of thrombin selectively cleave Arg-Gly bonds in fibrino-gen to from fibrin and fibrinopeptides A and B.

Jaffer et al. (2002) determined whether a human-targeted thrombin probe could be activated by murine thrombin in experimental thrombosis models. The researchers first verified probe activation by endogenous thrombin within mouse blood. Their in vitro experiments demonstrated substantial thrombin probe activation in accord with the known high degree of conservation between human and mouse thrombin (Banfield and MacGillivray, 1992). Banfield and Mac-Gillivray (1992) performed a cDNA sequence of the B chain of thrombin in nine vertebrate species (rat, mouse, rabbit, chicken, gecko, newt, rainbow trout, sturgeon, and hagfish). Of the 240 amino acids spanned in all the species compared, there is identity at 110 (45.8%) positions. When conservative changes are included, the amino acid similarity increases to 75%. The most conserved portions of the B chain are the active-site residues and adjacent amino acids, the B loop, and the primary substrate-binding region.

Although a few studies have shown the value of transmission electron microscopy (TEM) to investigate not only human, but also animal ultrastructure, not many researchers have used scanning electron microscopy (SEM) to study platelet and fibrin ultrastructure. Studies done include animals

ranging from rhinoceros, elephant, and bovine species (du Plessis and Stevens, 2002; du Plessis et al. 1996; Liu et al., 1998; Zucker-Franklin et al., 1985).

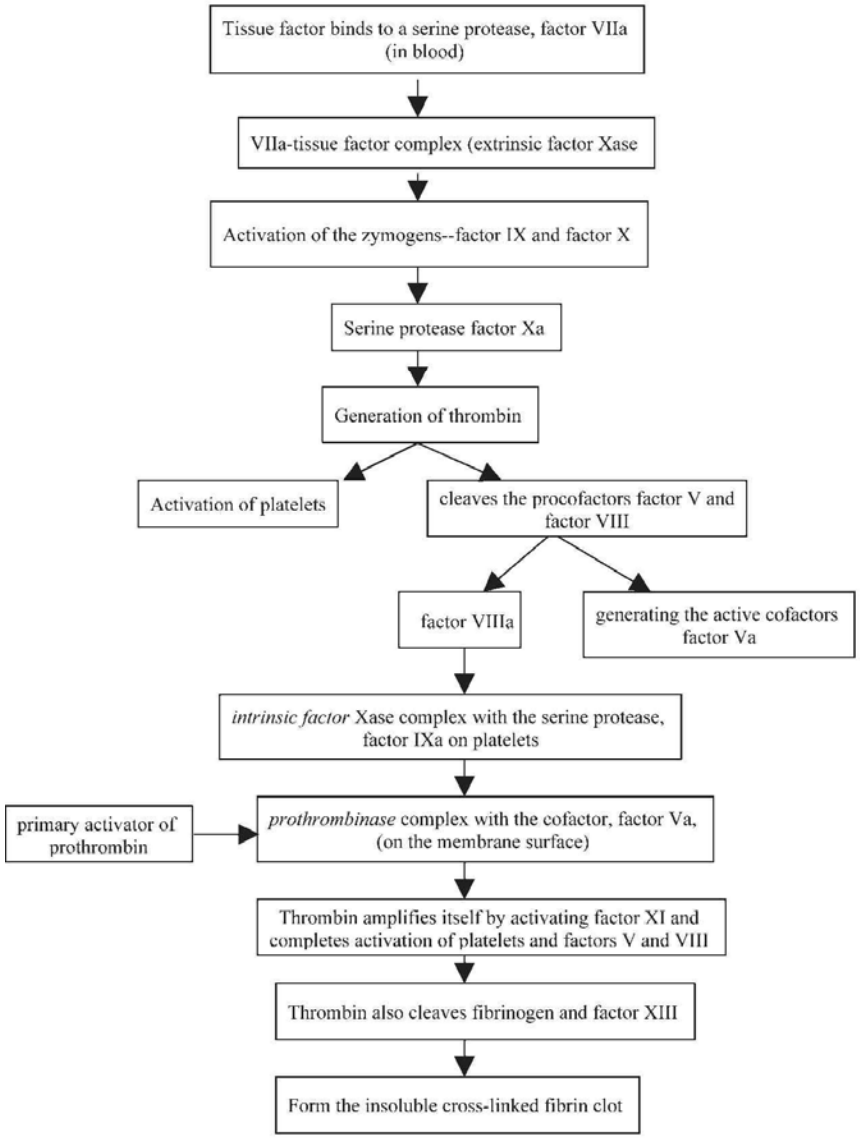


Chart 1. Simplified diagram of the intrinsic coagulation pathway.

Liu et al. (1998) studied the effect of procainamide on the ultrastructure of human blood platelets. They added arachidonic acid to procainamide-treated platelet-rich plasma to induce platelet aggregation and examined sections with the TEM. The authors found that procainamide inhibited the changes of ultrastruc-ture of blood platelet and releasing response and found changes of pseudopods, α-granules, dense granules, glycogens, open canalicular system, and dense tubular system.

Cheryk et al. (1998) studied the ultrastructure of platelets from the Asian elephant (Elephas maximus) before and after activation with the agonist platelet activating factor (PAF) using the TEM. The authors found that the unactivated platelets have a distinct ultrastructural appearance and that the cytoplasm contained large, randomly distributed granules with no internal cristae that typify the open canalicular system in other mammalian platelets. After PAF stimulation, large aggregates of platelets form, however, many platelets remained discrete entities. After aggregation some platelets seemed

swollen, and appeared if the granules were absent, whereas others retained their granules. Some platelets also developed gap-like features on the outer membranes.

du Plessis and Stevens (2002) studied internal platelet ultrastructure of the African elephant, using the light microscope, SEM, and TEM. Blood was collected from animals and blood smears were made, and showed that platelets have numerous pseudo-podia. SEM analysis showed that the platelets were pleomorphic.

du Plessis et al. (1996) compared buffalo (Syncerus caffer), bovine (Bos taurus), and human platelet morphology. After blood was drawn, platelet-rich plasma was obtained by centrifugation, and prepared for resin embedding and TEM analyses. The researchers found that human platelets have pseudopodia and intracellu-lar a-granules as well as surface-connecting canaliculi. Bovine platelets have a smooth surface, a-granules, as well as distinctive dense bodies in the cytoplasm and buffalo platelets closely resembled that of bovine platelets.

Ebbeling et al. (1992) studied the ultrastructure of human platelets. Platelet rich plasma (PRP) was cen-trifuged and a platelet pellet obtained, suspended in Hanks Balanced Salt Solution with 0.1% bovine serum albumin, and stimulated with thrombin and prepared for TEM. Results indicated that, after thrombin addition, platelets formed pseudopodia, and centralized their granules.

Little information is, however, available on platelet structure using SEM. Pretorius et al. (2006, 2007) studied the ultrastructure of fibrin networks in healthy humans as well as in a family with dysfibrinogenae-mia. PRP was obtained and platelets activated with human thrombin. Both platelet and fibrin networks were studied. Results indicated that thrombin-acti-vated platelets formed pseudopodia and platelets aggregates are visible. Also, thin minor and thicker major fibrin fibers are present. Fibers from dysfibrino-genaemia patients showed a typical stellate appearance and major fibers were longitudinally fused (Pretorius et al., 2006).

Because of lack of knowledge regarding the ultra-structure of platelets and fibrin networks of species other than humans (as studied using the SEM), the current study was done to determine the morphology of fibrin and platelets of mice and rabbits and compared with that of humans, using the SEM.

Materials And Methods

Preparation of Fibrin Clots

40 mL of blood was drawn in citrate vials from each healthy human donor; 100 µL of blood was drawn from 6 Balb-C mice and blood was pooled; 1,000 µL blood was drawn from each rabbit. Ethical clearance was obtained from the University Animal and Ethics committee. To both the mice and rabbit blood 11 µL citrate (0,109 M Trisodium citrate) for every 100 µL of blood, was added. Blood from the three groups was centri-fuged at 1,000 rpm for 2 min, to obtain PRP.

Human thrombin (provided by The South African National Blood Services) was used to prepare fibrin clots from human blood (Pretorius et al., 2006; Pretor-ius et al., in press) as well as blood from mice and rabbits. This thrombin is 20 U/mL and is made up in biological buffer containing 0.2% human serum albumin. When thrombin is added to PRP, fibrinogen is converted to fibrin and intracellular platelet components, e.g., transforming growth factor, platelet derived growth factor, and fibroblastic growth factor are released into the coagulum.

20μL of the PRP human donors was mixed with 20μL human thrombin. Because of the small volumes of blood from the mice and rabbits, 10μL of PRP was mixed with 10μL of human thrombin. The PRP and thrombin mix was immediately transferred with a pipette tip to a 0.2 lm millipore membrane to form the coagulum (fibrin clot) on the membrane. This millipore membrane was then placed in a Petri dish on filter paper dampened with PBS to create a humid environment and placed at 378C for 10 min. This was followed by a washing process where the millipore membranes with the coagula were placed in PBS and magnetically stirred for 120 min. This was done to remove any blood proteins trapped within the fibrin network (Pretorius et al., 2006).

Preparation of Washed Fibrin Clot for SEM

Washed fibrin clots were fixed in 2.5% glutaralde-hyde in Dulbecco's Phosphate buffered saline (DPBS) buffer with a pH of 7.4 for 1 h. Each fibrin clot was rinsed thrice in DPBS for 5 min before being fixed for 1 h with 1% Osmium tetraoxide (OsO_4.). The samples were rinsed thrice with distilled water for 5 min and. were dehydrated serially in 30, 50, 70, 90%, and three times with 100% ethanol. The SEM procedures were completed by critical point drying of the material, mounting, and examining the tissue with a JEOL 6000F FEGSEM.

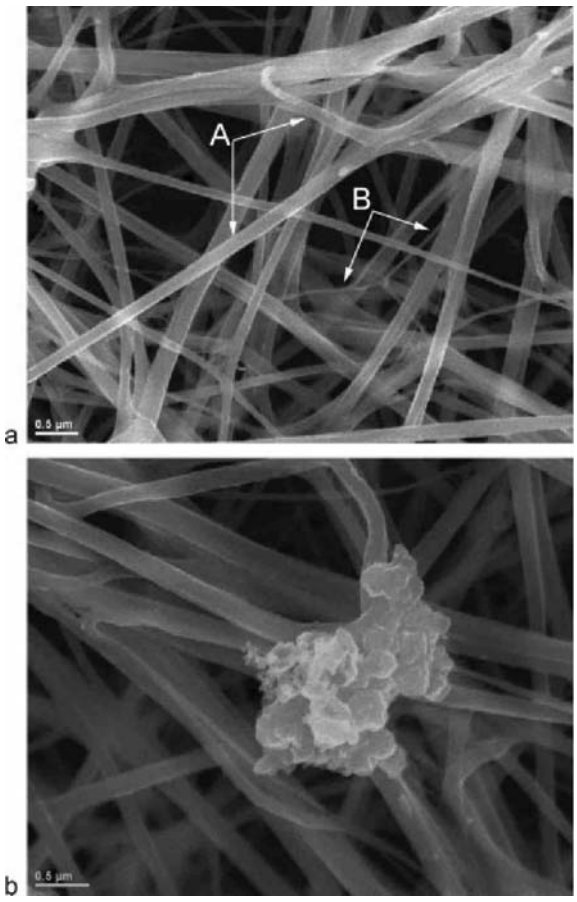
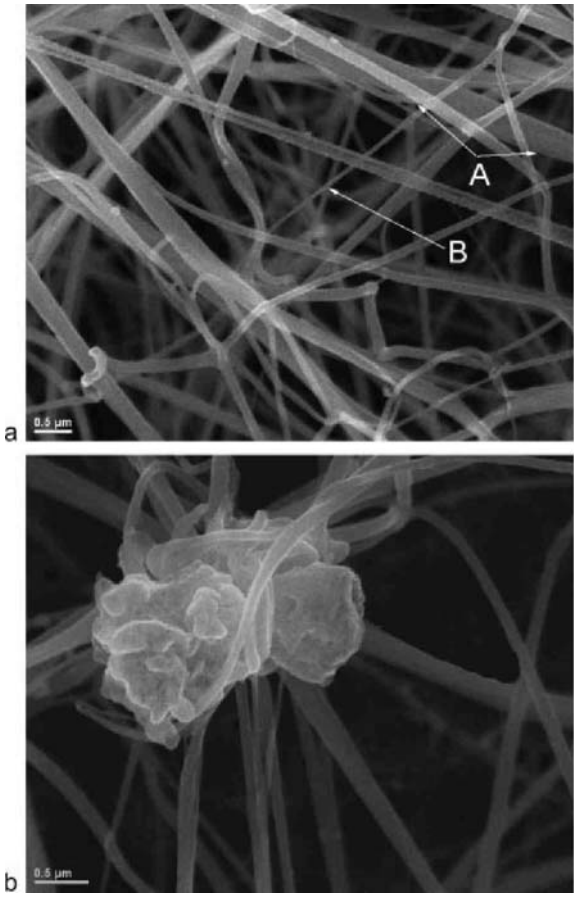


Fig. 1. (a) Human fibrin with major and minor fibers. Label A ¼ major, thicker fibers; Label B ¼ minor, thinner fibers. (b) Human platelet aggregate showing globular, pseudopodia-like morphology



.Fig. 2. (a) Rabbit fibrin network with major and minor fibers. Label A ¼ major, thicker fibers; Label B ¼ minor, thinner fibers. (b) Rabbit platelet aggregate showing globular, pseudopodia-like morphology.

Results And Discussion

Research discussed earlier suggests that the coagulation cascade of humans, rabbits, and mice are comparable, and that human thrombin can be successfully used to study fibrin formation in the three species. Therefore, this study used human thrombin to activate platelets and to study platelet and fibrin morphology.

Figure 1a and 1b show the fibrin network of a human fibrin clot and platelet. Figures 2a and 2b show the fibrin network and clot of rabbit blood and 3a and 3b that of mice fibrin and platelet.

Little differences were found between human and rabbit fibrin networks (Figs. 1a and 2a) as well as platelets (Figs. 1b and 2b). Human fibrin fibers show two distinct morphological types, namely major fibers and minor fibers (Pretorius et al., 2006). The major fibers tend to be most prominent, with the minor fibers much thinner and appearing between the major, thicker fibers (Fig. 1a, labels A and B). This was also found in the rabbit fiber network (Fig. 2a, labels A and B). Human fiber thickness varies from 0.15 to 0.35 lm, whereas rabbit major fiber thickness varies from 0.16 to 0.33 lm (Fig. 2a).

The mouse fibrin network varied considerably from that of both human and rabbit fibrin. Although the clot does indeed appear to have both thinner and thicker fibers (Fig. 3a, labels A and B), the thicker fibers have an approximate diameter of 0.03 µm, forming a very fine fibrin network. This fine network sometimes has a mat-like appearance (Fig. 3a, label C inside block). It was interesting to note

that, to take a SEM micrograph that show the fibers of the human/rabbit versus mouse fibrin, the SEM magnification for the human/rabbit clot was 6,000–10,000 x, while that of the mouse blood was between 30,000 and 40,000x.

Because PRP was used for all three types of clots, platelets were still present trapped between fibrin fibers, or lying on top of the network. Figures 1b, 2b, 2a, and 3b show the platelets of the three different species. Because of the coagulation process, single platelets are bound to each other to form aggregates of pla telets. Size of platelet aggregates therefore cannot be used to suggest differences between the three species. When comparing the ultrastructure of the platelet aggregates between the three species, no differences was noted. In all three species the aggregates have a rounded, globular pseudopodia-like appearance with the membranes of the aggregates forming a smooth surface. Previous TEM research also suggested that platelets form pseudopodia (du Plessis et al., 1996).

Both mice and rabbits are used frequently in testing of pharmaceutical products, in preclinical trial research. From the current research, it would seem as if the rabbit model provides a better option to study, particularly coagulation and hemostasis processes, as the fibrin network and the platelet aggregate ultra-structure seems comparable to that of humans. Also, both major and minor fiber thickness are well comparable to that of human tissue.

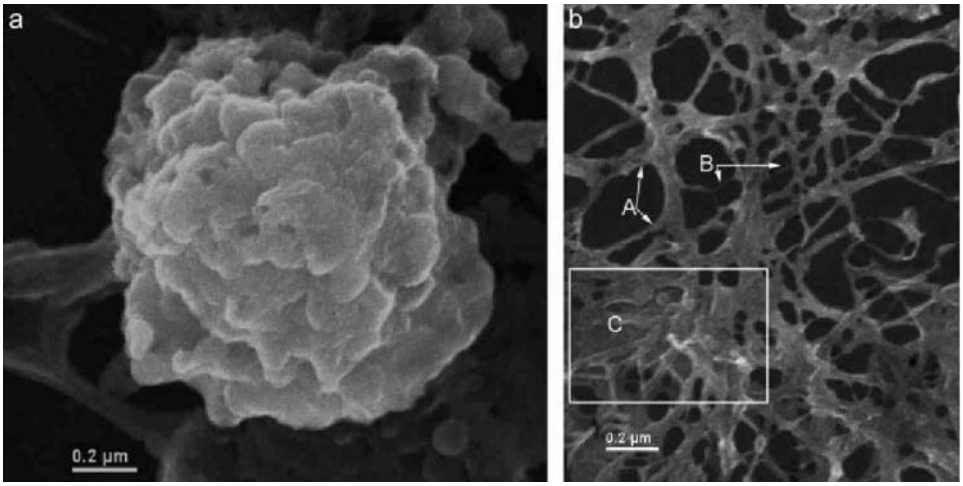


Fig. 3. (a) Mouse fibrin with fine major and minor fibers and areas of mat-like coverage. Label A ¼ major, thicker fibers; Label B ¼ minor, thinner fibers. Label C inside block ¼ mat-like fiber formation. (b) Mouse platelet aggregate showing globular, pseudopodia-like morphology.

Acknowledgments

We would like to acknowledge Prof. John Weisel from the University of Pennsylvania for the invaluable guidance regarding the preparation of the fibrin clot. We thank Prof. Franz and Sister Briedenhann from the Department of Surgery of the University of Pretoria for helping with the clot preparation. Special thanks to the Tommy Scanes from the South African National Blood Services for providing the human thrombin.

References

Banfield DK, MacGillivray RT. 1992. Partial characterization of vertebrate prothrombin cDNAs: Amplification and sequence analysis of the B chain of thrombin from nine different species. Proc Natl Acad Sci USA 89:2779–2783.

Bouma BN, Mosnier O. 2003. Thrombin activatable fibrinolysis inhibitor (TAFI) at the interface between coagulation and fibrinolysis. Pathophysiol Haemost Thromb 33:375–381.

Butenas S, Mann KG. 2002. Blood coagulation. Biochemistry 671:3–12.

Carmeliet P, Collen, D. 1998. Molecules in focus—Tissue factor. Int J Biochem Cell Biol 30:661–667.

Cheryk A, Gentry PA, Bast T, Yamashiro S. 1998. Alterations in the blood platelet morphology during aggregate formation in the Asian elephant Elephas maximus. J Zoo Wildl Med 29:177–182.

du Plessis L, Stevens K. 2002. Blood platelets of the African elephant. J Comp Pathol 127:208–210.

du Plessis L, Botha AJ, Stevens K. 1996. Ultrastructure of buffalo. Syncerus caffer, platelets: Comparison with bovine and human platelets. J Morphol 229:309–314.

Ebbeling L, Robertson C, McNicol A, Gerrard JM. 1992. Rapid ultra-structural changes in the dense tubular system following platelet activation. Blood 80:718–723.

Herd CM, Page CP. 1994. Pulmonary immune cells in health and disease: Platelets. Eur Respir J 6:1145–1160.
Jaffer FA, Tung CH, Gerszten RE, Weissleder R. 2002.

In vivo imaging of thrombin activity in experimental thrombi with thrombin-sensitive near-infrared molecular probe. Arterioscler Thromb Vasc Biol 22:1929–1935.

James HL, Larson PJ, Chao YB, Yan SB, Kim DJ. 1997. Isolation and characterization of mouse coagulation factor X—Biophysical and enzymological properties. Thromb Haemost 78:1049–1054.

Lui LP, Shan CW, Lui XH, Xiao HC, Yang SQ. 1998. Effect of procai-namide on ultrastructure of blood platelet in rabbits. Zhongguo Yao Li Xue Bao 194:376–379.

Marx PF, Wagenaar GT, Reijerkerk A, Tiekstra MJ, van Rossum AG, Gebbink MF, Meijers JC. 2000. Characterization of mouse throm-bin-activatable fibrinolysis inhibitor. Thromb Haemost 83:297–303.

McVey JH. 1999. Tissue factor pathway. Best Pract Res Clin Haema-tol 123:361–72.

Nemerson Y. 1988. Tissue factor and hemostasis. Blood 71:8.

Pretorius E, Briedenhann S, Marx J, Franz RC. 2006. Structural changes in the fibrin network of a Pretoria family with dysfibrino-genaemia: A scanning electron microscopical study. Ultrastruct Pathol 303:167–176.

Pretorius E, Briedenhann S, Marx J, Smit E, van der Merwe CF, Pieters M, Franz RC. 2007.

Ultra-structural comparison of the morphology of three different platelet and fibrin fiber preparations. Anatom Record 290:188–198.

Ruiz SM, Sridhara S, Blajchman MA, Clarke BJ. 2000. Expression and purification of recombinant rabbit factor VII. Thromb Res 98:203–211.

Zucker-Franklin D, Benson KA, Meyers KM. 1985. Absence of a surface-connected canalicular system in bovine platelets. Blood 65: 241–244.