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Age-related changes in fibrin networks and platelets of individuals over 75: a scanning electron microscopy study showing "thrombotic preparedness"

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Abstract During aging, changes in vasculature, haemostasis and endothelium, including alterations of platelets, coagulation and fibrinolytic factors, occur. Research has also reported that healthy, aged individuals have heightened coagulation enzyme activity, accompanied by signs of enhanced formation of fibrin and secondary hyperfibrinolysis. It is now believed that the impaired fibrinolytic potential in old age results in a condition that has previously been described as a systemic state of ''thrombotic preparedness''. This state is far out of proportion to the physiological needs of the person. In the current research we investigate whether this apparent changed thrombotic profile in healthy aged individuals (over the age of 75), is evident in their platelet and fibrin network ultrastructure, when compared to healthy individuals under 25 years. The main differences among young and older individuals were found in the fibrin network ultrastructure. It is concluded that with age, major fibers seem to become thinner and more sparsely arranged and that minor, thin fibers dominate it the coagulum, forming a fine netlike structure. At irregular intervals in the coagulum, thicker, fibrin fiber lattices are present; this is not found in healthy individuals. This might be due to the previously suggested enhanced fibrin formation and heightened coagulation enzyme activity. Here we therefore provide ultrastructural evidence for the thrombotic preparedness previously suggested after studying biochemistry of fibrinolysis and coagulation factors in the elderly.

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Introduction

During aging, changes in vasculature, haemostasis and endothelium, including alterations of platelets, coagulation and fibrinolytic factors, occur [1]. In old age an increasing number of healthy individuals have heightened coagulation enzyme activity, accompanied by signs of enhanced formation of fibrin and secondary hyperfibrinolysis [2]. Hume in 1961 already suggested that fibrin as well as fibrinolytic activity increased with age [3]. Currently, we know that in old age, we have an increase in plasma concentrations of fibrinogen, factor V, factor VII, factor VIII, factor IX, Von Willebrand factor, high molecular-weight kininogen and prekallikrein as well as fibrin D-dimer levels, plasmin–antiplasmin complex and thrombin activable fibrinolysis inhibitor (TAFI) [1, 2, 4–8]. Previous research also indicated that plasminogen activator inhibitor (PAI)-1, which is the major inhibitor of fibrinolysis, increases with aging [1, 9]. These changed concentrations may be the reason for the increase in the turnover of fibrin and may be associated with the age-related increases in endothelial disturbance and the prevalence of atherosclerosis [10]. Interestingly, an increase in Von Willebrand factor is an independent predictor of atherothrombotic disease [2]. However, according to Mari et al. (2008), high plasma levels of the coagulation activation markers in older populations do not necessarily suggest a high risk of arterial or venous thrombosis [2]. Platelet activity is also changed and could be due to a higher content of platelet phospholipids, suggesting an age-related increase in platelet transmembrane signalling or second messenger accumulation [1, 11].

Although there is this apparent change in haemostatic profile with increased age, these changes are not related to gender [3]. It is now believed that the impaired fibrinolytic potential in old age results in a condition that Gharacholou and Becker in 2009 described as a systemic state of ''thrombotic preparedness'' that is far out of proportion to the physiological needs of the person [12].

The question that now arises is whether this apparent changed thrombotic profile in healthy aged individuals (over the age of 75), is evident in their platelet and fibrin network ultrastructure, when compared to healthy individuals under 25 years of age. The current study therefore investigates the ultrastructure of platelets and fibrin networks of a 6 healthy, elderly individuals to determine if the thrombotic preparedness suggested by Gharacholou and Becker in 2009, is morphologically visible [12].

Materials and methods

Patients

Blood was drawn from six healthy individuals; with no known previous thrombotic events and not using anti-clotting medication (Ethical clearance was obtained from The Research Ethics Committee, Faculty Health Sciences, University of Pretoria, who complies with ICH-GCP guidelines and has US Federal wide Assurance; ethics clearance number: 151/2006, re-approved 2009). Their ages were: 78, 79, 84, 85, 86, 92. Six healthy, control donors (under the age of 25) were used in this study.

Preparation of fibrin clots

Fresh platelet-rich plasma from each donor was prepared by drawing 40 ml of blood. Blood was centrifuged at 1,000 rpm (maximum RCF = $17.523\times g$; $1{,}250\times g$) for 2 min. Human thrombin (provided by the South African National Blood Service) was used to prepare fibrin clots. The thrombin solution is at a concentration of 20 U/ml and is made up in a biological buffer containing 0.2% human serum albumin.

When thrombin is added to platelet-rich plasma, 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. 20 µl of the PRP was mixed with 20 µl of human thrombin on a 0.2 µm millipore membrane to form the coagulum (fibrin clot). This millipore membrane was then placed in a Petri dish on filter paper dampened with phosphate buffered saline (PBS) to create a humid environment and placed at 37°C 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 varying times (45, 90 and 120 min). This was done to remove any blood proteins trapped within the fibrin network [13, 14].

Preparation of washed fibrin clot for SEM

Washed fibrin clots were fixed in 2.5% glutaraldehyde in Dulbecco's Phosphate buffered saline (DPBS) buffer with a pH of 7.4 for 1 h. Each fibrin clot was rinsed thrice in phosphate buffer 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 drying of the material with hexamethyldisilazane [15], mounting and coating with ruthenium tetraoxide (SPI Supplies, West Chester USA).

Scanning electron microscope

A Zeiss ULTRA plus FEG-SEM with InLens capabilities, using nitrogen gas and ultra high resolution BSE imaging was used to study surface morphology of the samples. Micrographs were taken at 1 kV. This instrument is located in the Microscopy and Microanalysis Unit of the University of Pretoria, Pretoria, South Africa.

Results

Figure 1a shows a typical platelet aggregate and Fig. 1b shows fibrin networks as seen in healthy control subjects. Although new controls were used in the current study, the fibrin and platelet morphology of disease-free young, individuals have been well-reported on in the literature. Platelet aggregates appear bulbous, with pseudopodia arranged on the surface (white arrows). Typically, fibrin networks consist of robust, major, thick fibers (black arrows) and minor, thin fibers (white arrows), sparsely distributed among the major fibers.

Figure 2a and b show platelet aggregates seen in the aged sample. In all six individuals, it was found that coagulates contained aggregates well comparable to that of the younger sample. Figure 2a show such an aggregate (indicated by thick, white arrow block) that demonstrate the typical bulbous aggregate, with pseudopodia (thin, white arrows) arranged on the surface structure. However, the coagula of all six elderly individuals also contained smaller platelet masses that did not form a typical large,

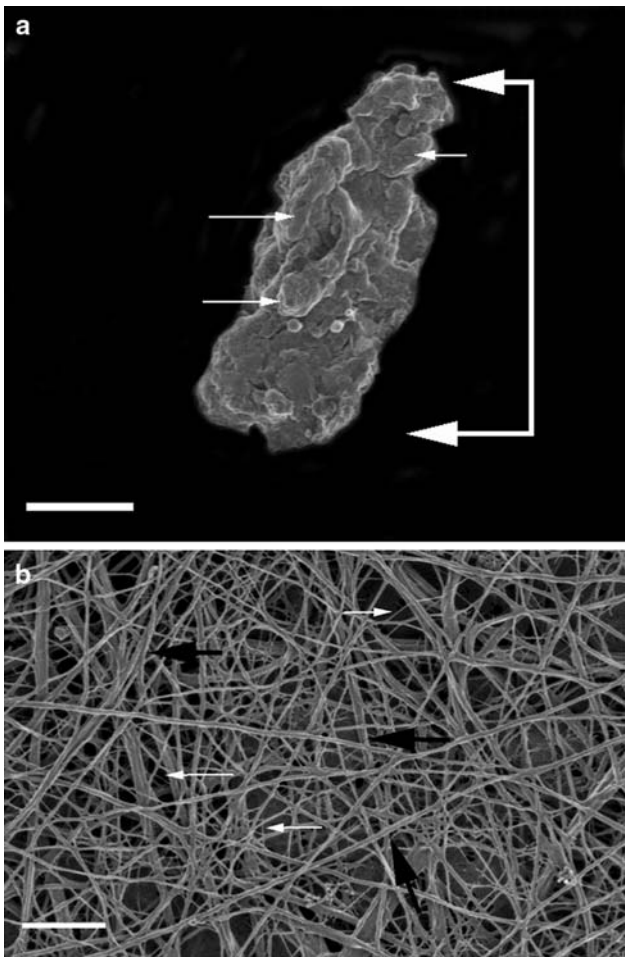

Fig. 1 **a** Scanning electron microscopy of platelet from a healthy control. *Arrow block* platelet aggregate. *Thin, white arrows* pseudopodia of aggregate. (Scale bar = 1 μm). **b** Scanning electron microscopy of fibrin fibers from a control. *Black arrows* major, thick fibers; *White arrows* minor, thin fibers (Scale bar = 1 μm)

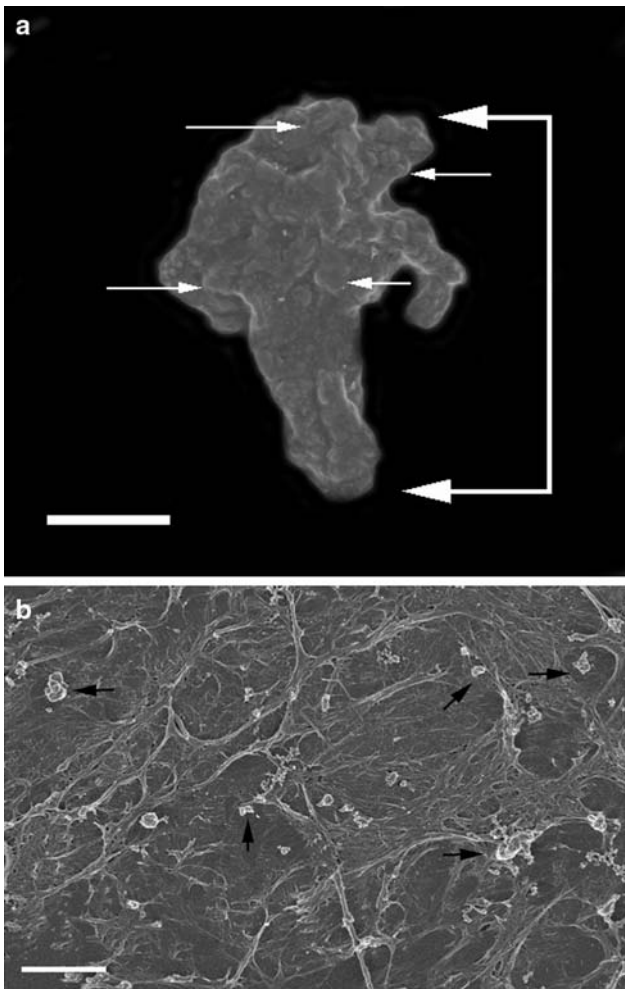

Fig. 2 **a** Scanning electron microscopy of a normal sized platelet aggregate from an aged individual. *Arrow block* platelet aggregate. *Thin, white arrows* pseudopodia of aggregate. (Scale bar = 1 μm). **b** Scanning electron microscopy of smaller platelet aggregates from an aged individual. *Black arrows* dispersed aggregates. (Scale bar = 1 μm)

bulbous structure. This is seen in Fig. 2b, where small platelet aggregates (black arrows) that were not fully activated to clump together, are seen.

Figure 3 a and b show fibrin networks typically noted in all six elderly individuals. Most of the coagula consisted of sparsely placed thick, major fibers (black arrows) with a fine, lattice of minor, thin fibers (white arrows) (Fig. 3a forming the majority of the coagula. Also present in the coagula, however, not frequently noted, are minor, fiber networks that form bulky, irregularly arranged lattices (thick, white arrows; Fig. 3b). Both the major and minor fibers seem much thinner and more flimsy than in healthy individuals [note scale 1 μm (Fig. 3a, b) versus 2 μm (Fig. 1b)]. In controls the major, thick fibers form the majority of the coagula. However, in the aged sample the minor fibers prevail. No lattice of minor fibers is seen in healthy, young individuals.

Discussion

In the current research we investigate the ability of platelets and fibrin networks of elderly, healthy patients to form coagula similar to that of younger, healthy individuals. Previously it was suggested that an impaired fibrinolytic state exists in the elderly, and Gharacholou and Becker in 2009 described this state as a thrombotic preparedness [12]. Here we investigate if this thrombotic preparedness can be visualized by studying coagula prepared by adding human thrombin to platelet rich plasma prepared form 6 healthy, elderly individuals and healthy donors, under the age of 25.

Many diseases like cancer, thrombotic disease, bleeding disorders, asthma and even conditions like HIV/AIDS are associated with ultrastructural changes in platelet and fibrin structure. Previous research suggests that these diseases

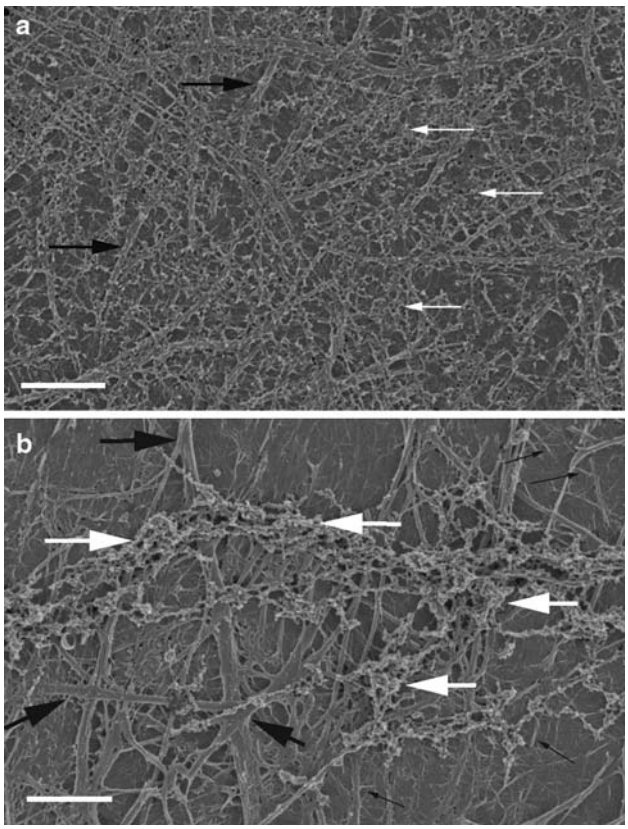

Fig. 3 **a** Scanning electron microscopy of flimsy fibrin fibers from an aged individual. *Black arrows* sparsely placed major, thick fibers; *White arrows* lattice of minor, thin fibers (Scale bar = 1 μm). **b** Scanning electron microscopy of flimsy fibrin fibers from an aged individual. *Thick, black arrows* sparsely placed major, thick fibers; *Thin, black arrows* minor, thin fibers; *Thick, white arrows* minor, thin fibers forming lattice of bulky, irregularly arranged areas (Scale bar = 1 μm)

show an altered platelet and fibrin structure morphology [16–18]. Previous research have also demonstrated the usefulness of ultrastructural analyses in the broadening of knowledge of disease patterns and suggests that information gained by using these techniques, may enhance treatment regimes [19].

Platelet aggregate ultrastructure seems consistent in younger and aged individuals. Control morphology is also consistent with results from previous studies of Pretorius and co-workers [16–19]. However, it is noted that some platelets do not aggregate into the typical, large, bulbous structures as is found in younger individuals. This changed morphology in some of the aggregates does probably not suggest impaired fibrinolysis, and could also not be due to excessive thrombotic activity. Rather, this might suggest impaired aggregation. During aggregation, platelets will release, on stimulus, factors contained in the alpha granules which facilitates aggregation, amongst others.

No apoptotic or necrotic aggregates were noted in the current aged sample. Previously, apoptotic platelet morphology has been shown in HIV patients [17]. Therefore, it is suggested that aggregation (on thrombin stimulation) may be slightly impaired in some platelets.

The main differences among young and older individuals were found in the fibrin network ultrastructure. It is concluded that with age, major fibers seem to become thinner and more sparsely arranged (Figs. 1a and 3a, b). Minor fibers seem to be more prevalent than in younger individuals, and therefore dominate the coagulum morphology. These minor, thin fibers, at irregular intervals, clump together to form minor fiber lattices (Fig. 3b). This might be due to the previously suggested enhanced fibrin formation and heightened coagulation enzyme activity.

Gharacholou and Becker in 2009 suggested that in aged individuals, a thrombotic preparedness is prevalent and we believe that we now presented ultrastructural evidence that this is indeed the case [12]. These changes are most prevalent in the structural organization of the fibrin networks.

Conclusion

Although most of the platelets aggregates appear normal, it seems as if the thrombin did not adequately activate the attachment process in some platelets; hence we see some smaller aggregates spread throughout the fibrin network. These elderly individuals were chosen because they have had no previous thrombotic event and also do not use medication for related conditions. It would be interesting to investigate the ultrastructure of elderly individuals that indeed suffers from conditions affecting haemostasis.

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References

1. Franchini M (2006) Hemostasis and aging. Crit Rev Oncol Hematol 60(2):144–151
2. Mari D, Coppola R, Provenzano R (2008) Hemostasis factors and aging. Exp Gerontol 43(2):66–73
3. Hume R (1961) The relationship to age and cerebral vascular accidents of fibrin and fibrinolytic activity. J Clin Pathol 14(2):167–171
4. Al-Zahrani H, Lowe GDO, Douglas JT, Cuschieri R, Pollock JG, Smith WCS (1992) Increased fibrin turnover in peripheral arterial disease: comparison with a population study. Clin Hemorheol 12:867–872
5. Tracy RP, Bovill EG, Fried LP (1992) The distribution of coagulation factors VII and VIII and fibrinogen in adults over 65 years. Results from the Cardiovascular Health Study. Ann Epidemiol 2:509–519
6. Giansante C, Fiotti N, Cattin L, Da Col PG, Calabrese S (1994) Fibrinogen D-dimer and thrombin-antithrombin complexes in a

random population sample: relationships with other cardiovascular risk factors. Thromb Haemost 71:581–586
7. Sagripanti A, Carpi A (1998) Natural anticoagulants, aging, and thromboembolism. Exp Gerontol 33:891–896
8. Schatteman KA, Goossens FJ, Scharpe SS, Neels HM, Hendriks DF (1999) Assay of procarboxypeptidase U, a novel determinant of the fibrinolytic cascade, in human plasma. Clin Chem 45: 807–813
9. Wilkerson WR, Sane DC (2002) Aging and thrombosis. Semin Thromb Hemost 28:555–567
10. Lee AJ, Gerald F, Fowkes R, Lowe GDO, Rumley A (1995) Determinants of fibrin D-dimer in the Edinburgh Artery Study. Arterioscler Thromb Vasc Biol 15:1094–1097
11. Bastyr EJ, Kadrofske MM, Vinik AI (1990) Platelet activity and phosphoinositide turnover increase with advancing age. Am J Med 88:601–606
12. Gharacholou SM, Becker RC (2009) Hemostasis and thrombosis in older adults. J Thromb Thrombolysis 27(2):249–251
13. 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 fibre preparations. Anat Record 290:188–198
14. Pretorius E, Oberholzer HM, Smit E, Steyn E, Briedenhann S, Franz RC (2008) Ultrastructural changes in platelet aggregates of HIV patients: A scanning electron microscopy study. Ultrastruct Pathol 32:75–79
15. Araujo JC, Téran FC, Oliveira RA, Nour EAA, Montenegro MAP, Campos JR, Vazoller RF (2003) Comparison of hexamethyldisilazane and critical point drying treatments for SEM analysis of anaerobic biofilms and granular sludge. J Electron Microsc 52(4):429–433
16. Pretorius E, Bornman MS, Reif S, Oberholzer HM, Franz RC (2009) Ultrastructural changes of platelet aggregates and fibrin networks in a patient with renal clear cell adenocarcinoma: a scanning electron microscopy study. Microsc Res Tech 72: 679–683
17. Pretorius E, Oberholzer HM, Smit E, Steyn E, Briedenhann S, Franz RC (2009) Investigating the ultrastructure of platelets of HIV patients treated with the immuno-regulator, Canova: a qualitative scanning electron microscopy study. Histol Histopathol 24:399–405
18. Pretorius E, Oberholzer HM (2009) Ultrastructural changes of platelets and fibrin networks in human asthma: a qualitative case study. Blood Coagul Fibrinolysis 20(2):146–149
19. Pretorius E (2009) The role of platelet and fibrin ultrastructure in identifying disease patterns. Pathophysiol Haemost Thromb