Scanning electron microscopy investigation of fibrin networks after thermal injury

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Injury due to burning is known to impact on coagulation and haemostasis by disturbing the coagulation cascade and is also associated with impaired fibrinolysis. Also, venous thrombosis, pulmonary embolism and hypercoagulability are common during thermal injury. Using a Wistar albino rat model, we investigated in this study whether burn injury affects the ultrastructure of the fibrin networks. A typical fibrin network will contain mostly major, thick fibres with minor, thin fibres distributed amongst them. We found that the clot architecture changes after burn injury, showing more prominent minor, thin fibres in a netted appearance. Also, the clot showed areas of matted fibrin. We suggest that the thrombotic events associated with burn injury are due to the thickened and netlike areas formed when thrombin activates the coagulation cascade. This is due to impaired fibrinolysis activities, causing the resulting fibrin clots not to be successfully disseminated. Small fragments of these netted, clumped areas may therefore break loose and lead to thrombotic events after burn injuries. The current study therefore provided morphological evidence for thrombotic events associated with burn injury.

Introduction

Injury due to burning is known to impact on coagulation and haemostasis (Lavrentieva *et al.* 2008) by inducing subclinical disseminated intravascular coagulation. Fibrinolysis, in particular, is affected, with fibrinolytic plasma parameters showing an activation of fibrinolysis 2 hours after burn wound creation, followed by suppression in fibrinolysis after 24 hours and up to 10 days post-burn (Fang, Ding & Kong 1997). Such suppression of fibrinolysis may protect fibrin deposited in the wound, which could be important in wound healing. Fang *et al.* (1997) suggested that the suppressive factors of fibrinolysis may be due to enhanced activities of proteins such as plasminogen activation inhibitor-1 (PAI-1) and alpha 2-antiplasmin after injury. Also, burn injury causes an acquired deficiency of the plasma protein antithrombin III (ATIII), a natural anticoagulant that leads to a high incidence of hypercoagulability and prevalence to thrombosis in patients (Kowal-Vern *et al.* 2000). ATIII is a natural anticoagulant and the most important inhibitor of blood coagulation owing to its effect on thrombin (Kowal-Vern *et al.* 2000; Penner 1995). Venous thrombosis is associated with low plasma levels of ATIII known to occur in burn patients; also, pulmonary embolism, hypercoagulability, adult respiratory distress syndrome, infection and multiple organ failure are common in thermal injury (Darling *et al.* 1996; Gando, Tedo & Kubota 1992; Geerts 1994; Kowal-Vern *et al.* 2000; Kowal-Vern *et al.* 1992; Penner 1995). Ravindranath *et al.* (2004) also found that plasma tissue factor pathway inhibitor (TFPI) levels are significantly decreased 24 h after a burn, while thrombin activatable fibrinolytic inhibitor (TAFI) levels are significantly increased 24 h and 72 h after a burn.

The question that now arises is whether burn injury has an impact on fibrin network architecture in circulating plasma, and whether a local burn injury will change the clot ultrastructure. A Wistar rat burn wound model was subsequently used to study the ultrastructure of fibrin networks.

Materials and methods
Choice of burn wound model

Fifteen Wistar albino rats (between 200 g and 250 g each), which were maintained at the University of Pretoria Biomedical Research Centre, were used in this study (three untreated controls and 12 burn injury animals). The rats were fasted for 12 hours before thermal injury; however, they had free access to water. The rats were kept in a room with a 12-hour alternating light and dark cycle and room temperature was kept constant at 22 °C. The animals were fed a standard rat diet, provided with water *ad libitum* and were housed in individual polycarbonate cages. All experimental protocols complied with the requirements of the University of Pretoria Animal Use and Care Committee (Ethical Clearance number: H021/08).

Burn wound creation

On the day of burn wound creation, the rats were anaesthetised with isoflurane and directly injected with the analgesic Tramadol™. Wound creation commenced only 15 min after injection of the analgesic to allow it to take effect. The dorsum of each rat was shaved and then exposed to a 1 cm x 1 cm brass block, pre-heated to 95 °C in a hot water bath, for 10 s. The brass block was placed on the dorsum of the rats using gravity only, which resulted in partial thickness skin burns. A physiological saline solution was then administrated intraperitoneally (25 mg/kg) to prevent dehydration of the animal.

Immediately after thermal injury, all wounds were dressed with a primary gauze dressing and Opsite™ as the secondary dressing. To prevent the rats from interfering with the healing process the secondary dressing was covered with a third bandage dressing and fastened with Elastoplast around the edges.

All animals received another injection of Tramadol 1 h after thermal injury. In addition, all animals received a four-hourly subcutaneous injection of analgesics for the remainder of the study.

Preparation of fibrin clots

On day 7, during termination, blood was drawn from each animal and 11 µL of citrate was added to every 100 µL of blood drawn. The blood from each animal was kept separately and studied individually. Blood was centrifuged at 1250 rpm for 2 min to obtain platelet-rich plasma (PRP).

Thrombin (provided by the South African National Blood Services) was used to prepare fibrin clots (Pretorius, Ekpo & Smit 2007; Pretorius, Oberholzer & Smit 2009a; Pretorius et al. 2009b). The thrombin (20 U/mL) was prepared in a biological buffer containing 0.2% human serum albumin. When thrombin is added to PRP, fibrinogen is converted to fibrin and intracellular platelet components; for example, transforming growth factor, platelet-derived growth factor and fibroblastic growth factor are released into the coagulum.

A volume of 10 µL of mouse PRP was mixed with 10 µL thrombin. The PRP–thrombin mix was immediately transferred by pipette to a 0.2-µm Millipore membrane to form the coagulum (fibrin clot). The Millipore membrane was then placed in a Petri dish on filter paper dampened with phosphate-buffered saline (PBS) (to create a humid environment) and kept at 37 °C for 10 min. The coagula-containing membranes were then washed (by placing them in PBS and magnetically stirring for 20 min) to remove any blood proteins trapped within the fibrin network.

Preparation of washed fibrin clot for scanning electron microscopy

Washed fibrin clots were fixed in 2.5% gluteraldehyde in Dulbecco's PBS (0.075 M) at a pH of 7.4 for 1 h. Each fibrin clot was rinsed three times in phosphate buffer for 5 min before being fixed for 1 h with 1% osmium tetraoxide. The samples were rinsed three times for 5 min with distilled water and were then dehydrated serially in 30%, 50%, 70% and 90% ethanol, and three times with 100% ethanol. The scanning electron microscopy procedures were completed by drying the samples with hexamethyldisilazane (Araujo et al. 2003) before being mounted, followed by coating samples with ruthenium tetraoxide and examining the tissue with a Zeiss ULTRA Plus FEG scanning electron microscope.

Results and discussion

Burn injury results in an inflammatory response and there is a synergistic response between the resulting inflammation and coagulation systems (Park et al. 2008; Sherwood & Toliver-Kinsky 2004). White blood cell distribution changes during the inflammatory response.

As mentioned previously, haemostasis changes during burn injury. After burn injury there is a brief hypocoagulation phase, followed by hypercoagulation owing to intensified procoagulant activity. These steps, in turn, result in depression of fibrinolysis (Kirillov & Alekaeva 1975). Both thrombotic and fibrinolytic pathways are directly triggered proportionally to the extent of the burn injury (Bartlett et al. 1981; Opal 2000; Wells, Sissons & Hasleton 1984). Although this has been researched thoroughly in the past, we do not know what the impact of burn injury is on the ultrastructure of the fibrin networks. From previous research it is known that inflammation causes fibrin networks to present with a changed morphology (Oberholzer, Vieira & Pretorius 2009; Pretorius et al. 2007). Typically, control fibrin networks consist of major, thick fibres forming the bulk of the clot, with minor, thin fibres sparsely distributed among them (Pretorius et al. 2009a; Pretorius et al. 2009b). We also know that this arrangement is found in humans, rabbits and mice. However, the thickness of rodent fibrin differs considerably from that of humans (Pretorius et al. 2009b). Inflammation in humans and mice has previously been shown to look similar, although the fibre thickness varies. Pretorius and Oberholzer (2009) showed that inflammation in humans and mice show major, thick fibres covered by a thin, matted layer of minor, thin fibres.

In the current study, control rat fibrin networks were studied and were found to appear similar to those of BALB/c mice; major, thick fibres with minor fibres distributed in between (Figure 1). In the burn wound injury animals in this study, fibrin fibres showed a typical inflammatory profile 7 days after injury (Figure 2a–c). Figure 2a shows major, thick fibres (thick, white arrow) and more prominent minor, thin fibres (thin, white arrows), as well as areas where fibrin has a netted appearance (black arrow). Figure 2b shows areas of the clot where thickened, matted fibrin is present (white arrow). Some areas of the clot also have a netted appearance (Figure 2c: white arrows). This changed morphology suggests that thermal injury affects not only the local area where the wound was created, but also the broader inflammatory processes and ultimately coagulation and haemostasis.
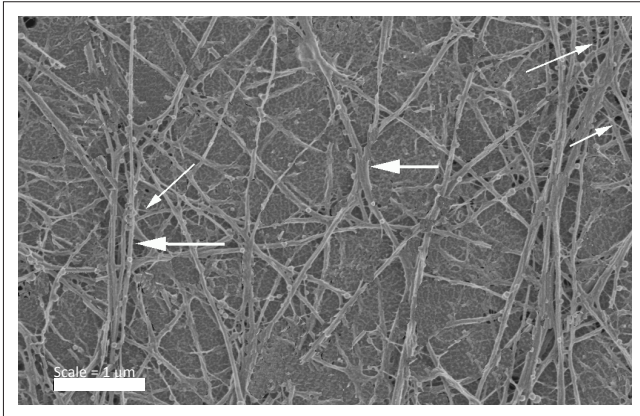
Thick, white arrows indicate major, thick fibres; thin, white arrows indicate minor, thin fibres.

FIGURE 1: Control rat fibrin network.
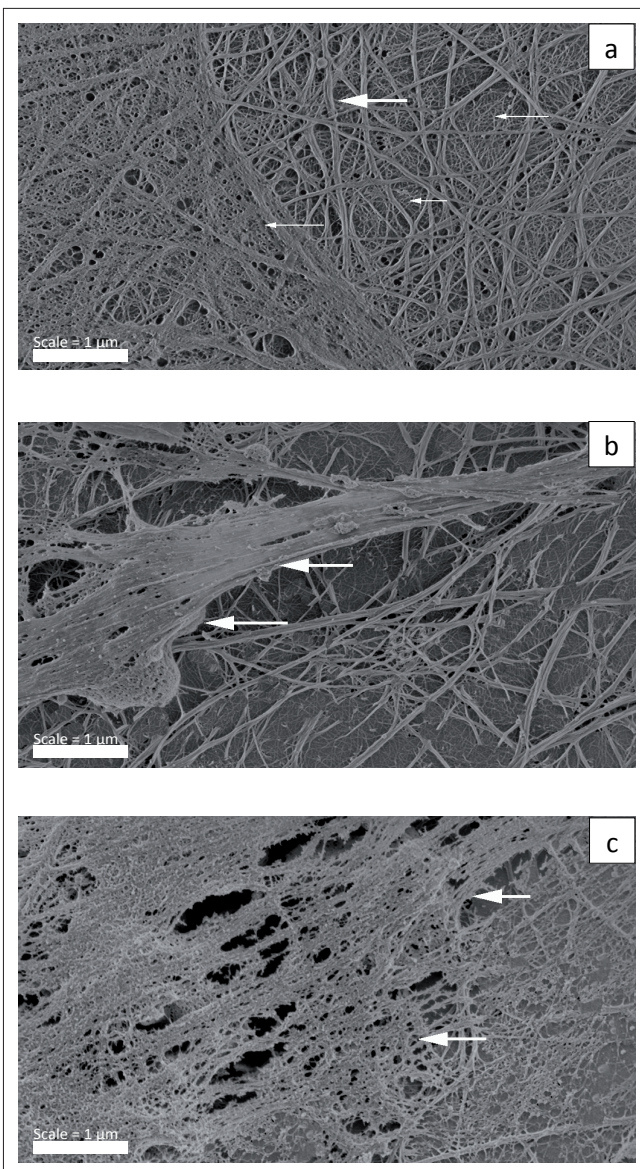
a, thick, white arrow indicates major, thick fibres; thin, white arrows indicate minor, thin fibres with netted appearance; black arrow indicates matted fibrin.
b, white arrows indicate thickened, matted fibrin.
c, white arrows indicate minor, thin fibres with netted appearance.

FIGURE 2: Rat fibrin network after thermal injury (day 7).

Ravindranath *et al.* (2004) mentioned that burn injury disturbs the coagulation cascade and thrombotic process in the procoagulant pathway by impairing fibrinolysis. Also, venous thrombosis, pulmonary embolism and hypercoagulability are common in thermal injury (Darling *et al.* 1996; Gando *et al.* 1992; Geerts 1994; Kowal-Vern *et al.* 2000; Kowal-Vern *et al.* 1992; Penner 1995). Here we suggest that the thrombotic events associated with burn injury are due to the thickened, netlike areas formed when thrombin activates the coagulation cascade. Because of impaired fibrinolysis activities, the resulting fibrin clots can then not be disseminated successfully. Small fragments of these netted, clumped areas may therefore break loose and, owing to already insufficient fibrinolysis activity, cause thrombotic events after burn injuries.

Conclusion

This study investigated whether burn injury affects the ultrastructure of fibrin networks. Results obtained with a rat burn wound model showed morphological changes to the structure of fibrin clots that formed after burn injury. These changes are likely due to impaired fibrinolysis, which explains thrombotic events associated with burn injury.

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