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Ultrastructural investigation of the time-dependent relationship between breast cancer cells and thrombosis induction

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ABSTRACT

Thromboembolic complications are a common cause of death in breast cancer patients. The *in vivo* relationship between coagulation factors and circulating tumours is a multifaceted one, with tumour cells implicated in thrombocytosis and platelets associated with coagulation-mediated metastasis. Platelets and fibrin networks are known to be morphologically altered in patients with cancer, and their relationship with breast cancer cells themselves may increase thrombosis susceptibility. This was investigated *in vitro*, assessing such morphological alterations through the establishment of a MCF-7 breast cancer cell co-culture system with blood plasma. Co-culture duration ranged from zero to thirty minutes, with five-minute intervals, in order to assess the time-dependent ultrastructural conformations of platelet and fibrin networks, using scanning electron microscopy. It was found that enhanced coagulability was related to co-culture duration. Changes in platelet and fibrin network morphology from normal were visible as early as five minutes in co-culture with MCF-7 cells. With longer co-culture duration thrombosis-linked variation in structural configuration was intensified, including advanced platelet aggregation and adherence characteristics, compression of fibrin networks into plaques of increased density, and upsurge of microparticulate extrusion implicated in amplifying procoagulant events during the metastatic process. These results confirm that cancer cells are stimulators of coagulation even in an *in vitro* system and breast cancer patients may become increasingly susceptible to thrombotic-related consequences with increased exposure to tumour cells, especially during metastasis.

1. Introduction

Thromboembolic complications are the second most common cause of mortality in breast cancer patients (Falanga and Donati, 2000; Nash et al., 2002). The *in vivo* interaction between circulating tumours and coagulation factors is complex and reciprocal – with platelet activation and aggregation implicated in facilitating coagulation-mediated metastasis, and tumour-derived cytokines and growth factors implicated in thrombocytosis (Bambace and Holmes, 2011). Specifically, following intravasation into the vascular system, tumour cell-secreted factors induce platelet activation and aggregation, thereby protecting tumour cells from high velocity shear forces and immunosurveillance (Bambace and Holmes, 2011). Thus malignancy itself is associated with increased thromboembolic risk (Falanga and Donati, 2000; Nash et al., 2002). Furthermore, as depicted in Fig. 1, inflammation (leukocyte involvement) and platelet activation are closely interlinked (van der Spuy and Pretorius, 2013), and though not in the scope of this paper, inflammation is imperative in malignant processes (Jurasz et al., 2004).

Morphological alterations in platelet and fibrin network morphology have long been associated with thrombotic risk and such architectural shifts are evident in disease conditions such as diabetes, stroke and cancer (Pretorius et al., 2009, 2011a,b). It is thus understood that the *in vivo* relationship between breast cancer cells and coagulation factors may lead to changes in platelet and fibrin network morphology as well as function, increasing susceptibility to thrombosis. It is known that cancer cells are able to produce platelet agonists including thrombin, which not only induce platelet activation (Picker, 2011) but are a requirement for fibrin network formation. During normal haemostasis, the generation of a fibrin clot and subsequent fibrinolysis forms part of the coagulation process. However, in disease states including thromboembolic ischemic stroke, fibrin clot structure and fibrinolysis can be dysregulated (Pretorius et al., 2011b). The higher the amount of coagulation agonists produced and the longer the exposure dura-

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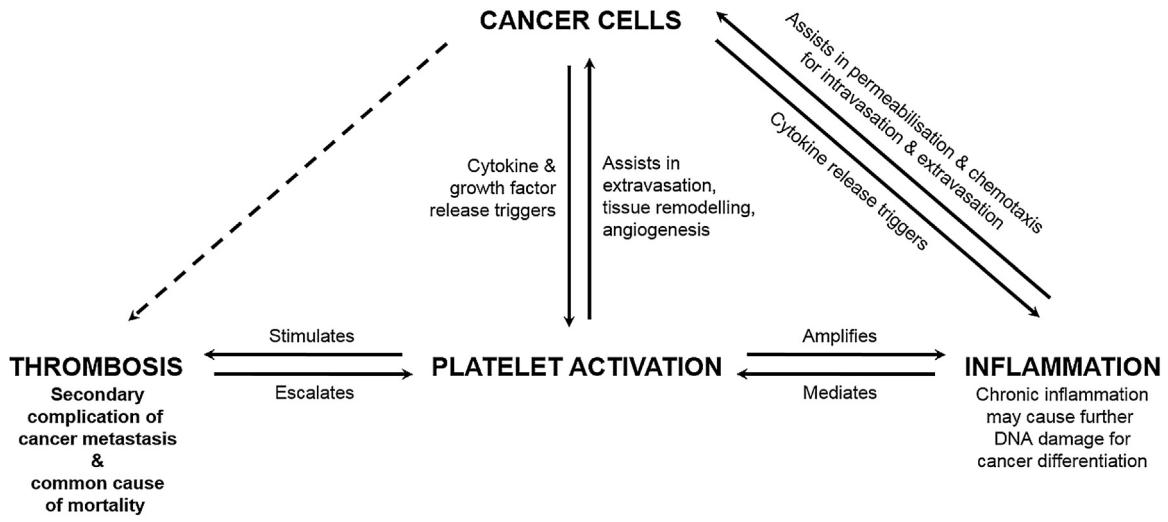

Fig. 1. Platelet activation is central to cancer progression. Platelet activation amplifies the inflammatory response, stimulates thrombosis processes, assists in the extravasation of circulating cancer cells, and has a role in the establishment of new tumour growth.

tion to these agonists, the further and more rapidly are platelets expected to advance through phases of activation, aggregation and full adherence.

This study was designed to investigate the *in vitro* environment in order to ascertain how soon tumour cells affect coagulation ability or propensity for thrombosis, as a starting point for further studies into their relationship.

2. Materials and methods

A co-culture system was established, culturing MCF-7 luminal phenotype breast cancer cells with the blood plasma of seemingly healthy female individuals. Applicable exclusion criteria included smoking, contraceptive use or pregnancy, and a history of autoimmune disease or cancer. The experiment was repeated in triplicate (conducted separately) with the blood of two different volunteers per experiment (n = 6). Ethical clearance was obtained from the University of the Witwatersrand's Human Ethics Committee (M081036 and M140155).

MCF-7 cells were plated at a concentration of 1×10^5 cells per coverslip in a 24-well plate and incubated for 24 h at 37 °C and 5% CO_2 in normal DMEM media (Dulbecco's Modified Eagles Medium, Lonza, Verviers, Belgium; BE12-604F), with 10% heat-inactivated FBS (Foetal Bovine Serum, Biowest, South America; S1810-500) and 0.1% P/S (Penicillin/Streptomycin, Highveld Biological, RSA; 214). Normal media was replaced with Phenol Red Free (PRF)-DMEM media (Lonza, Verviers, Belgium; BE12-115F) with 10% Dextran Coated Charcoal (DCC)-stripped FBS, 0.1% P/S and L-glutamine, and incubated for 48 h at 37 °C and 5% CO_2 for cell cycle synchronization (Gil et al., 2013).

Peripheral blood was obtained *via* venepuncture into citrate-containing vacutainers (Lasec, South Africa). Diagnostic data suggests that citrated platelets – in plasma which is separated from the whole blood whence it is obtained by centrifugation – do not activate upon standing even after 1500*g* centrifugation for up to 15 min, and such separated plasma remains stable for diagnostic haemostasis testing up to 24 h in an unrefrigerated state (Favaloro et al., 2012). However, we have also shown that centrifugation at 400×g for the generation of PRP, may induce early activation as evidenced by the loss of CD62p (p-selectin) containing-microparticles (Augustine et al., 2016). As such in this study, platelet-rich plasma (PRP) was obtained by centrifuging whole blood for 5 min at 1000 rpm (200×*g*). Co-cultures were thus implemented by exposing PRP to cells for periods of zero (control – no exposure of plasma to cells) up to 30 min with intervals of 5 min, after which platelet and fibrin network coagula were prepared on glass coverslips for scanning electron microscopy (SEM).

MCF-7 cells were not prepared for scanning electron microscopy in this experiment, as it was designed specifically to determine co-culture duration for further experiments. Platelet coagula were prepared using only 20 μl of platelet rich plasma, whereas fibrin coagula were prepared through activation of 15 μl platelet rich plasma with 5 μl of 20 U/ml human thrombin (SANBS, South Africa (van der Spuy and Pretorius, 2013)). Prepared coverslips were incubated on 0.1 M phosphate buffered saline (PBS)-dampened filter paper for 2 min to ensure adherence, after which a washing process followed where the coverslips were submerged in PBS on a shaker for 20 min to ensure removal of trapped proteins. This was followed by primary fixation in 2.5% formaldehyde/glutaraldehyde solution for 15 min, three rinses in PBS, secondary fixation in 1% osmium tetroxide (OsO_4) for 15 min, three rinses in PBS and then serial dehydration in 30%, 50%, 70%, 90% and three times absolute ethanol. SEM procedures were completed by drying the samples with hexamethyldisilazane (HMDS), mounting on aluminium stubs and coating by carbon evaporation.

The ultrastructural alterations in platelet and fibrin network morphology were then assessed with a ZEISS ULTRA plus FEG Scanning Electron Microscope at the University of Pretoria's Microscopy and Microanalysis Unit in South Africa. Accelerating voltage was set at 1 kV for high quality surface analysis and samples imaged with the In-lens secondary electron detector. For each of the coagulum coverslips prepared, ten low magnification images were acquired to give an overall perspective of the level of coagulation, and ten high magnification images were acquired for clear visualization of morphological characteristics. Therefore, for each time point, 60 high magnification images were taken of platelets and fibrin networks, respectively, and a representative image was selected for depiction in this report.

3. Results

Control preparations of PRP not exposed to tumour cells showed typical morphology for platelets and fibrin networks. Platelets in the zero co-culture control groups displayed discoid to slightly rounded platelets with smooth membrane surfaces and little to no pseudopodia. No aggregates were present in these groups. Fibrin