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Scientists in Training abstracts

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Scientist in Training - 1

Fibrin Film and its Interactions with Red Blood Cells and Platelets

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Background: Thrombus composition is heterogeneous consisting of fibrin, red blood cells (RBCs), platelets, and leukocytes. Recent studies have shown that films, a sheet-like structure, develop on the surface of clots exposed to air, therefore playing a protective role. Evidence is emerging that fibrin films are also present in intravascular thrombi; however, little is known about the role of these films and their interactions with RBCs and platelets.

Aims: The aims of this study are to investigate the effect of haematocrit on fibrin films and the effect of fibrin films on platelets spreading.

Methods: Blood samples were obtained from healthy volunteers, and haematocrit (RBC concentration) was adjusted to 10, 30, 50, 70%. The effect of haematocrit on fibrin film formation/coverage was investigated by scanning electron microscopy. Platelets spreading on the surface of clots made from purified fibrinogen in the presence or absence of film was investigated by laser scanning confocal microscopy.

Results: We demonstrated that fibrin films covering the blood clot also appeared to adopt an intermediate form where the film is not continuous, but instead includes many pores or holes. All blood clots within the range of haematocrit used developed fibrin film; however, blood clots formed with 10% haematocrit showed less fibrin film coverage than those with a higher haematocrit (30-70%). The spreading of platelets on clots with film was reduced compared to clots without film or fibrin directly coated onto the coverslip.

Summary: The formation of fibrin film is influenced by haematocrit levels. Our data suggest that RBCs may support fibrin film coverage. The area of platelet spreading was reduced on clots with film, suggesting that fibrin films impede platelet spreading. These findings may have important implications for clot formation and thrombus stability.

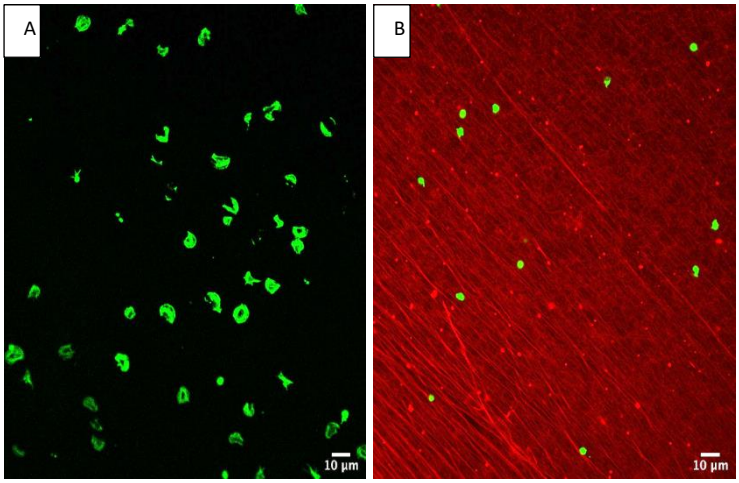

Figure. Platelets spreading on coverslips coated with fibrin (A) and fibrin clot with film (B). AlexaFluor^{488} phalloidin was used for platelets (green). AlexaFluor^{594} fibrinogen was used to visualise fibrin clot (red). Images were taken by Laser scanning confocal microscopy. Magnification: x40.

Scientist in Training - 2

Neutrophils and NETs Produce Clots with Different Types of Fibrin Fibers

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Background: Previously we showed neutrophil extracellular traps (NETs) promoted clotting independently of FXII, FXI and FVII, and contributed to the formation of a denser clot architecture that is more resistant to lysis. We also found neutrophils induced blood clotting in a different manner than NETs, specifically mediated by FXI. Hitherto, effects of neutrophils and NETs on fibrin fiber structure and fibrinopeptide release are unknown.

Aims: To compare effects of differentiated PLB-985 cells (a neutrophil-like cell model), human neutrophils and their NETs on the structure of fibrin fibers, and investigate the effects of neutrophils on fibrinopeptide release.

Methods: Human neutrophils were isolated from whole blood by standard density gradient centrifugation method. PLB-985 cells were differentiated in 1.25% (v/v) DMSO. Neutrophils and differentiated PLB-985 cells were stimulated by PMA to generate NETs. Scanning electron microscopy (SEM) was used to image the fiber structure of clots. Atomic force microscopy (AFM) was used to detect the diameter of PLB-985 NET fibers. ELISAs were carried out to analyze fibrinopeptide A and B in neutrophil-induced clots.

Results: Differentiated PLB-985 cells, PLB-985 NETs and human NETs induced the formation of a network structure of clots (Fig 1), and significantly increased the diameter of fibrin fibers. Human neutrophils increased the release of fibrinopeptide B in plasma, but the structure of human neutrophil-induced clots was lacking an overall "scaffold" structure (Fig 1). AFM data showed that the thickness of NET fibers was variable but thicker than a single DNA helix (2 nm), suggesting that NET fibers are made up of multiple strands of DNA.

Conclusions: Differentiated PLB-985 cells, PLB-985 NETs and human NETs increased fibrin fiber thickness, which may enhance the clot stability. Human neutrophils on the other hand failed to produce normal fibrin fibers.

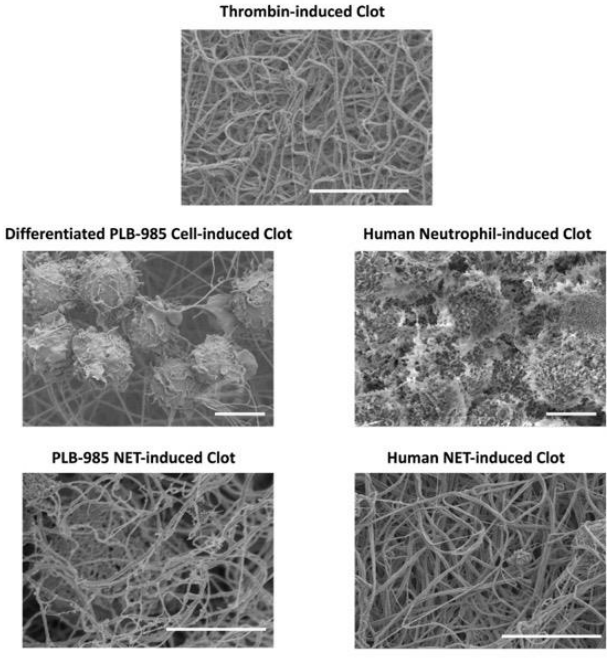

Figure 1. SEM images of plasma clots. Clots were induced by differentiated PLB-985 cells, PLB-985 NETs, human neutrophils and human NETs, respectively. 1 U/ml Thrombin-induced clot was used as a control. Final concentrations: Plasma (diluted 1:3), 10 mM $CaCl_2$. Scale bars are 5 µm.

Scientist in Training - 3

HSP47 regulates collagen structure and synthesis by megakaryocytes.

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Collagen accounts for 30% of total protein mass in the body and HSP47 is known to be a collagen chaperone, escorting procollagen from the ER to the golgi. We have previously reported that platelets possess HSP47, which is secreted to the cell surface whereupon in positively influences the ability of platelets to interact with and respond to collagens at sites of injury. Megakaryocytes (MKs) are known to secrete extracellular matrix proteins including type IV collagen, fibronectin, and laminin to maintain the bone marrow environment. Collagen overproduction/mis-production are associated with a number of pathologies including fibrosis associated with myeloproliferative disorders. This led us to hypothesise that HSP47 may contribute to MK function and collagen production.

Using a MK specific knockout model of HSP47, primary MKs were isolated and cultured in the presence of TPO and hirudin. Mature MKs were separated from smaller less mature MKs using a BSA gradient and used to measure the impact of HSP47 on MK development and function: the ability to become polyploid, measured using propidium iodide and analysed by flow cytometry; to produce proplatelets and the ability to synthesize collagen measured using a fluorogenic reagent to detect oligopeptides following enzymatic degradation of used cell media.

Deletion of HSP47 caused no change in circulating platelet numbers, red and white blood cell counts suggesting the bone marrow niche is not grossly affected by the genetic modification. Indeed ploidy was comparable with that of control mice. Consistent with this, proplatelet formation by mature MKs was also unchanged. Collagen production from cultured primary murine HSP47 deficient MKs was reduced by 38% compared with control megakaryocytes ($P<0.05$). These findings are consistent with megakaryocytes contributing to collagen present in the bone marrow and therefore that HSP47 may be a therapeutic target for myeloproliferative disorders to prevent fibrosis of the bone marrow.

Scientist in Training - 4

Shear-induced platelet reactivity in native blood assessed at the point-of-care correlates with thrombin generation in patients with myocardial infarction

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Background

Shear-induced platelet activation (SIPA) at sites of high-grade coronary stenoses have been implicated in the pathomechanism of occlusive coronary thrombus formation leading to acute coronary syndrome (ACS). SIPA likely leads to thrombin generation and therefore, measuring shear-induced platelet reactivity may identify patients who may benefit from potent and tailored antithrombotic medication. The ATLAS ACS 2-TIMI 51 study which assessed the prognostic benefit of adding low dose indirect thrombin inhibitor, rivaroxaban, in ACS observed a reduction in thrombotic events, but at the expense of increased bleeding, highlighting the need to tailor thrombin inhibition.

Aim

We aimed to assess shear-induced platelet reactivity at the bedside of ACS patients and correlate this with thrombin generation.

Methods

Non-anticoagulated venous blood from patients admitted acutely with ACS (n=87) was assessed using the point-of-care Global Thrombosis Test (GTT) and thrombin generation was subsequently measured in citrated plasma using the Calibrated Automated Thrombogram (CAT). The GTT is an automated technique, which assesses native whole blood under high shear to measure the time taken for occlusive thrombus formation (occlusion time, OT). A short OT represents high platelet reactivity. Thrombin generation was assessed using the CAT and related to OT.

Results

We found an inverse correlation between OT and peak thrombin generation (r=-0.388, p=0.001) and velocity (r=-0.564, p<0.001). Division of patients into quartiles (Q1-4) according to OT demonstrated that a shorter OT was related to increased endogenous thrombin potential (Q1: 1469[342-1784] vs Q4: 1009[148-1524]nM.min, p=0.042), shorter lag time (Q1: 6.3[5.2-7.5] vs Q4: 8.3[6.9-10.0]min, p=0.002) and higher velocity index (Q1: 72[31-97] vs Q4: 25[13-45]nM/min, p=0.003).

Conclusions

Patients with ACS show enhanced shear-induced thrombotic occlusion at point-of-care which correlates with more rapid and increased thrombin generation. Future studies are required to determine whether the GTT has utility to identify patients at increased risk of future thrombotic events who may benefit from tailored anticoagulation.

Scientist in Training - 5

Plasminogen Activator Inhibitor-1 and its cofactor vitronectin induce a hypofibrinolytic state in COVID-19 disease which correlates with disease severity

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Background: COVID-19 disease arises from infection with severe acute respiratory cornonavirus-2 (SARS-CoV-2). Patients with severe COVID-19 exhibit a unique coagulopathy characterised by elevated D-dimer levels, fibrin deposition in the lung, and a thrombotic incidence of approximately 30%, indicating catastrophic derailment of the haemostatic system.

Aim: To investigate whether the coagulopathy in patients with COVID-19 arises due to derailment of the fibrinolytic arm of the haemostatic pathway.

Methods: We performed a dual-centre study of 139 patients presenting with symptomatic COVID-19 and compared to 24 patients with non-SARS-CoV-2 respiratory infection and 30 healthy controls. We evaluated fibrinolytic biomarkers, including plasminogen activator inhibitor 1 (PAI-1), tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), vitronectin, thrombin activatable fibrinolysis inhibitor (TAFI), plasminogen. Diagnostic biomarkers including, fibrinogen, C-reactive protein (CRP), D-dimer and inflammatory cytokines were quantified. Clot lysis time was evaluated using a turbidimetric approach and plasma clot structure was visualised by confocal microscopy.

Results: We found elevated levels of acute phase proteins, specifically, fibrinogen, CRP and D-dimer in COVID-19 patients compared to healthy controls. Notably, only CRP was significantly elevated in COVID-19 patients compared to other respiratory infections. Analysis of fibrinolytic proteins revealed significantly elevated levels of tPA and TAFI in patients with COVID-19 compared to healthy controls. Importantly, PAI-1 antigen, activity and its cofactor, vitronectin, were also significantly increased in COVID-19 patients compared to healthy controls and non-COVID-19 respiratory infection. PAI-1 levels were also found to correlate with inflammatory cytokines (IL-1β, IL-8 and TNF-α). Plasmin-generation and clot lysis reveal significantly attenuated fibrinolytic activity in patients with COVID-19 compared to healthy volunteers.

Discussion: This study reveals that the hypofibrinolytic state in COVID-19 patients is induced by high levels of PAI-1 antigen and activity, which correlate with its cofactor, vitronectin, and inflammatory cytokines. This study highlights the potential prognostic power of the fibrinolytic protein PAI-1 in the development of severe COVID-19.