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Summer students abstracts

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Summer student abstract - 1

Developing a model of trauma induced coagulopathy using a functional plasmin generation assay

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Background

Worldwide, traumatic injury accounts for 4.9 million deaths every year[1]. Uncontrolled bleeding accounts for 25% of all injury-related deaths[2,3] and 40-80% of potentially preventable deaths[4]. Trauma haemorrhage is exacerbated by the presence of trauma induced coagulopathy (TIC), a multi-phenotypic disease state that comprises disorders of coagulation and inflammation, and describes the overall failure of the coagulation system to maintain haemostasis after major injury. TIC is associated with significantly poorer outcomes, including increased need for major haemorrhage therapy and early transfusion requirements, development of organ failure and 3-4 fold increased risk of death[5,6].

TIC occurs in the acute phase after severe tissue injury and major haemorrhage[7], and enforces a hyperfibrinolytic state during the initial stages of TIC that is exacerbated by fibrinogen depletion[8,9]. Substantial gaps remain in identifying the pathophysiological mechanisms of TIC and to do this we require appropriate experimental models and a more detailed understanding of the pathophysiology. Most studies of trauma have focussed on quantifying coagulation and fibrinolytic proteins at a single time point or have used thromboelastography parameters as an indicator of clot strength[10]. These studies do not provide an accurate measurement of functional fibrinolytic potential.

Aim

To determine the impact of restoring fibrinogen levels on plasmin generation in a model of TIC.

Experimental plan

We have developed an *in vitro* model of TIC utilising fibrinogen deficient plasma (FDP) to recapitulate fibrinogen loss during trauma haemorrhage[11]. This project will investigate how two sources of fibrinogen replacement therapy, fibrinogen concentrate (Fg-C) and cryoprecipitate, alter plasmin activation during TIC.

Cryoprecipitate is rich in coagulation factors that are not present in Fg-C[12], which may influence the rate of plasmin activation. Furthermore, the majority of trauma patients will be administered tranexamic acid (TXA) as part of their pre-hospital care, an anti-fibrinolytic drug that functions by inhibiting plasmin activation[13]. We propose to study the sensitivity of our TIC model to TXA inhibition and determine whether the source of fibrinogen replacement is impacted by TXA. An in house plasmin generation assay and a chromogenic substrate specific to plasmin will be used to quantify plasmin generation over time. The influence of TXA on fibrinolysis will be further studied using a turbidity assay to quantify clot lysis times.

To further investigate any differences in clot strength and stability between the two sources of fibrinogen replacement; Fg-C and cryoprecipitate; we propose to form Chandler model thrombi[14] using our TIC model. Fg-C and cryoprecipitate will be added to FDP at increasing concentrations, and thrombi dissolved for analysis of α_2-antiplasmin incorporation and evidence of fibrin cross-links using western blot.

The levels of plasmin-α_2AP (PAP) complexes are elevated in trauma and have been a focus of many studies, demonstrating the significant generation of plasmin in TIC[15,16]. This project will develop and optimise an *in vitro* model of TIC to enable further study and understanding into the role of the fibrinolytic system in the pathophysiology of TIC. The data obtained will also provide further insight into differences in clot strength and stability between the fibrinogen sources and whether one is superior for management of traumatic haemorrhage.

During the placement, the student will learn a range of haematological and biomedical experimental techniques and how to perform data analysis using dedicated software such as Graph Pad Prism and Shiny App[17]. The placement will also provide the student the opportunity to gain laboratory experience that will complement his medical degree.

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Summer student abstract - 2

Investigating the role of Gasdermin D in platelet function and thrombosis

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Background

Deep vein thrombosis (DVT) is a common cause of morbidity, and is associated with excess long-term mortality, partly through complications such as pulmonary embolism. Traditionally, stagnant venous blood flow, vessel wall injury, and hypercoagulability of blood are understood to be the principal causes of DVT. In recent decades, it has been shown that development of DVT is preceded by local inflammation, a process termed immunothrombosis. In particular, the recruitment of leukocytes and platelets to the venous wall is critical for the onset of thrombosis.

Nod-like receptor protein (NLRP)-3 inflammasomes are molecular complexes primarily focused on the transformation of caspase-1 and -11 into their active forms, which leads to cleavage and activation of inflammatory cytokines IL-1β and IL-18. Although inflammasomes are classically described as forming in immune cells, lately, their presence and activity have also been demonstrated in platelets.

We have recently shown that inflammasomes and neutrophil extracellular traps (NETs) co-operatively promote DVT by supporting local inflammation at the vessel wall, and that pharmacological inhibition of caspase-1 protects mice against venous thrombosis (1). This establishes a key role for inflammasomes and caspase-1 activation in the development of DVT.

Gasdermin D is a protein employed by leukocytes that forms part of a pathway downstream to $NLRP_3$ inflammasome and inflammatory caspase activation. Cleaved gasdermin D forms membrane pores, a process designated as pyroptosis, acting as a conduit for IL-1β secretion from the cytosol.

IL-1β has previously been shown to be crucial to the development of DVT whilst platelets contain all the machinery for IL-1β synthesis. Platelets have been shown to contain $NLRP_3$ inflammasomes and its assembly can be triggered by NETs or their main component histones (1). Recently, we have demonstrated the presence of gasdermin D in both murine and human platelets and shown that high dose histone 3 stimulation can cause cleavage of gasdermin D into its active form (unpublished data). However, cleavage of gasdermin D seems to be specific to histone 3 as stimulation using classical platelet activators (namely thrombin, collagen, U46619/PGH2, ADP and adrenaline) fails to do so (unpublished data).

Aims

To extend our previous observations that gasdermin D is present in both human and murine platelets, in this project we aim to:

1. Elucidate whether gasdermin D has a role in platelet functions.
2. Investigate the role of gasdermin D in arterial and venous thrombosis.

The proposed study will contribute to our understanding of fundamental principles of platelet physiology and functioning, shed light on the role of platelets in inflammation and their cooperation with the immune system, and potentially identify novel target(s) to prevent thrombosis. Furthermore, we anticipate these targets to be remarkably safe because it is unlikely that the gasdermin D system is implicated in physiological bleeding arrest.

Experimental Plan

Two main experimental approaches will be used to achieve our project aims:

1. Platelet function testing Inhibition of gasdermin D in isolated human platelets with Disulfiram followed by platelet function tests.

   Experimental outcomes: aggregation, spreading, granule secretion, microvesicle secretion, substance uptake from plasma.

2. Characterization of experimental thrombi/thrombus development derived from our existing models of arterial and venous thrombosis following *in vivo* inhibition of gasdermin D by Disulfiram.

   Experimental outcomes: thrombosis incidence; weight and length of thrombi; histological and flow cytometric characterization of thrombi; real time evaluation of processes preceding thrombus development using intravital microscopy.

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Summer student abstract - 3

Investigating Sex-Specific Differences in the Antithrombotic Capacity of Endothelial Cells

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Background

Cardiovascular disease (CVD) it is the leading cause of death in women, and in 2019 was responsible for 35% deaths [1]. There are many factors which contribute to inequity between men and women in the detection and management of cardiovascular disease [1], a major factor being a paucity of studies investigating the sex-specific differences in the pathophysiology of CVD. The majority of deaths attributed to CVD are caused by occlusive thrombi, which develop in diseased arteries and obstruct blood flow to the myocardium or brain, resulting in myocardial infarction or stroke respectively. In healthy blood vessels, the vascular endothelium plays a pivotal role in preventing thrombosis and maintaining haemostasis. Firstly, it acts as a physical barrier, shielding platelets from prothrombotic subendothelial matrix proteins. It also releases nitric oxide (NO), prostacyclin (PGI_2) and CD39/CD73 which inhibit platelet activation [2]. Endothelial cells express a plethora of natural anticoagulants including thrombomodulin (TM), tissue factor pathway inhibitor (TFPI), endothelial receptor for activated protein C (APC), and heparan sulphates which bind antithrombin III, and it actively promotes fibrinolysis through endogenous expression of plasminogen activators tPA and uPA [3,4] ensuring that any clots that do form are rapidly broken down.

Damage to the vascular endothelium as observed in CVD results in a phenotypic switch whereby the protective effects of endothelial cells are lost and replaced with proinflammatory and prothrombotic processes. Activated endothelial cells express tissue factor (TF) stimulating coagulation, Weibel–Palade bodies are released, containing P-selectin and VWF, which mediate platelet-leukocyte interactions and platelet adhesion to the vessel wall. The potent vasoconstrictor and platelet agonist, thromboxane A2 (TXA2) is generated and plasminogen activator inhibitor-1 (PAI-1) is expressed [3,4], all of which promote thrombus assembly. Currently it is not known whether the antithrombotic protection provided by a healthy endothelium in equitable in men and women. It is also not known whether there is dimorphism in the phenotypic switch evoked by vascular insult. Whole genome expression analysis of male and female HUVECs has demonstrated intrinsic sex differences in immune related genes and differential transcriptional changes in response to shear stress [5]. Intrinsic transcriptomic sex differences in endothelial cells have also been associated with coronary artery disease targets in GWAS studies [6]. If sex-specific differences in the endothelial-mediated regulation of thrombosis exist, these may contribute to poorer outcomes observed in women with CVD. It would also provide further evidence that sex should be treated as a biological variable and disease management stratified according.

Aim: The aim of this study is to determine whether there are any sex related differences in the antithrombotic capacity of endothelial cells.

Objectives: To address the aim we will:

1. Determine whether there are any sex-specific differences in plasma levels of key endothelial derived regulators of coagulation (thrombomodulin, TFPI, VWF) and fibrinolysis (tPA, uPA and PAI-1).
2. Determine whether there are any correlations between plasma levels of sex hormones and endothelial derived regulators of coagulation and fibrinolysis measured in Objective 1.
3. Determine whether there are any sex-differences in the surface expression of TF, TFPI and thrombomodulin on endothelial microvesicles (EMVs).

4. Determine whether there are any correlations between plasma levels of sex hormones and TF, TFPI and thrombomodulin expression on EMVs (Objective 3).

Methods: The study will be performed using plasma and serum samples already collected from a cohort of 108 healthy subjects (52 males; 56 females), where full blood counts were performed, health and lifestyle questionnaires were carried out, biometric data was obtained and platelet aggregation assays performed (ethical approval by Manchester Metropolitan University Ethics Committee)

Multiplex and ELISA: Commercially available multiplex assays (R&D Systems) will be used to measure the levels of thrombomodulin, TFPI, vWF, tPA, uPA and PAI-1, following manufacturer's instructions. The levels will then be compared between plasma samples from male donors (n=52) and those from female donors (n=56) to determine whether there are any significant sex-specific differences between circulating levels of any of the thrombotic regulators. To investigate whether there is an association between sex hormones and the plasma levels of any of the endothelial derived regulators, the level of testosterone and 17-β oestradiol will be quantified by ELISA (Abcam) following manufacturer's instructions, and regression analysis performed to determine whether there are any significant correlations.

Analysis of endothelial microvesicles.

Endothelial microvesicles will be analysed in citrated plasma by flow cytometry. Gating will be performed using anti-CD31 and anti-CD42b antibodies [7]. Microvesicles positive for CD31 but negative for platelet marker CD42b will be selected as the endothelial derived microparticle population. Using anti-TFPI, anti-TF, anti-thrombomodulin fluorescently labelled antibodies, the surface expression levels present on EMVs will be measured as an indicator of endothelial expression, and this will be compared between the sexes to determine whether there is a statistically significant difference. Regression analysis will be performed against EMV expression levels of the thrombotic regulators and testosterone and 17-β oestradiol levels to identify if there are any correlations with sex hormones.

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