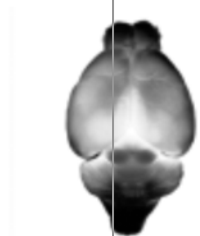
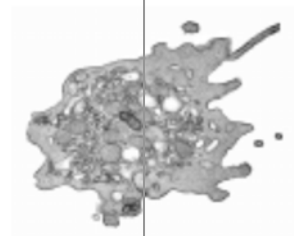


Australian Centre
For Blood Diseases:

*Research
Projects*

2009 - 2010





Thrombosis Research Unit

Investigation of the effects of new anti-cancer agents on platelet function: *A role for apoptosis in regulating platelet reactivity*

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Evasion of apoptosis is a hallmark of cancer. One of the primary ways in which cancerous cells evade apoptosis is by upregulating members of the Bcl-2 family of proteins, important prosurvival proteins that protect against apoptosis. Overexpression of Bcl-2 proteins is commonly associated with several blood cancers, including lymphoma and multiple myeloma. Thus, a significant effort is currently underway to develop small molecule Bcl-2 inhibitors that bind to the BH3 domain of Bcl-2 proteins (BH3 mimetics) to induce apoptosis. Preliminary evidence has demonstrated that BH3 mimetics elicit tumour regression in several models of cancer, as well as potentiating the effects of commonly used anticancer drugs. An unexpected finding with the BH3 mimetics is that they cause a rapid loss of circulating platelets (thrombocytopenia), suggesting that the Bcl-2 family of proteins plays an important role in regulating platelet survival. These findings have raised the possibility that BH3 mimetics may not only be useful for the treatment of cancers, but may also be used to reduce platelet counts in patients with thrombocytosis (*a common disorder which leads to increased numbers of platelets in the circulation and an increased blood clotting tendency*).

The importance of the Bcl-2 family of proteins in regulating platelet function has not been defined. Using genetic mouse models deficient in prosurvival or proapoptotic proteins, Bcl_{XL} and Bak/Bax, this project aims to identify the importance of the apoptotic machinery in modulating platelet functional responses. This project will involve a broad range of experimental techniques including molecular biology, flow cytometry and western blotting, confocal and scanning electron microscopy, as well as a host of cell biological assays. Ultimately these studies will help establish whether BH3 mimetics can be used safely and effectively for the treatment of a range of cancers and blood clotting disorders.

Investigating new approaches to dissolve blood clots

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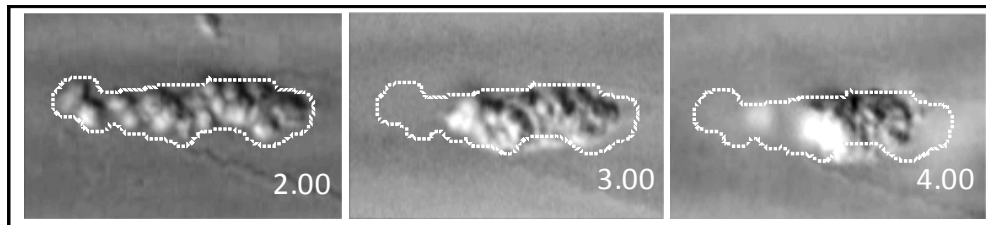
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Blood platelets play a critical role in the development of occlusive arterial blood clots (thrombi), precipitating diseases such as heart attack and ischaemic stroke. The rapid reperfusion of occluded blood vessels to minimise tissue death is a key treatment goal in patients suffering heart attack and stroke, with the administration of thrombolytic therapy an important means of establishing reperfusion. This is usually achieved through administration of fibrinolytic agents modelled on tissue-type plasminogen activator (tPA), which can effectively restore blood flow if administered within 12 hours of symptom onset. However, thrombolytic therapy is not without its limitations, with lysis resistant blood clots, as well as hemorrhage presenting as major complications.

Our laboratory is investigating novel ways of improving the removal of occlusive blood clots. Fundamental to blood clot formation is the ability of platelets to develop contractile forces, which allow them to pull on the fibrin scaffolding surrounding the blood clot, shrinking it in size in a process known as clot retraction. Recent studies in our laboratory have discovered a new platelet contraction process, ‘*primary thrombus contraction*’, causing tight packing of platelets in a blood clot, enabling the clot to avoid detachment caused by the shearing forces of blood flow (Ono *et al*, 2008). We will also explore the possibility that inhibition of this process may provide a new way in which to loosen blood clots, facilitating thrombolysis and thereby promoting their removal (blood clot lysis or thrombolysis).

These studies will involve the use of *in vitro* flow-based assays, genetic mouse models and state-of-the-art imaging systems (confocal microscopy), complemented with *in vitro* biochemical analysis of blood clot lysis. These studies will not only provide important insight into our understanding of blood clot formation, but may also lead to new approaches to regulate the size and stability of blood clots forming in the body, providing major clinical benefit in the delivery of thrombolytic therapy (blood clot removal).



This figure depicts the process of ‘*Primary thrombus contraction*’, causing the tight-packing of a platelet thrombus forming under blood flow over time.

References:

Akiko Ono, Erik Westein, Sarah Hsiao, Warwick S Nesbitt, Simone M Schoenwaelder* and Shaun P Jackson*, [*Equal senior author]. Identification of a unique platelet contractile mechanism regulating primary hemostasis and thrombus growth. *Blood*, 112(1):90-99, 2008.

Editorial: Alan T. Nurden. Thrombus stability at the vessel wall, *Blood*, 112(1):4-5, 2008.

Identification of a new family of proteins regulating blood clotting Role of Dok2 in regulating platelet function

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Blood platelets play a pivotal role in the development of cardiovascular diseases, such as heart attack and stroke. Physiologically, these cells rapidly adhere to sites of blood vessel injury to initiate blood clotting reactions that prevent excessive blood loss and vascular repair, however in diseased atherosclerotic blood vessels, excessive platelet accumulation leads to the formation of pathological blood clots (thrombosis) that prevent blood flow to vital organs. One of the critical receptors on the surface of platelets promoting thrombosis is the adhesion receptor integrin $\alpha_{IIb}\beta_3$; with deficiency of this receptor leading to a severe bleeding disorder termed *Glanzmann thrombasthenia*. Inhibitors of integrin $\alpha_{IIb}\beta_3$ are used in the clinic to prevent the development of heart attacks in high risk patients and while much is known about the mechanisms that *activate* this receptor, much less is known about the *negative* regulators of integrin $\alpha_{IIb}\beta_3$ adhesive function. In recent years, a new family of proteins, termed Dok adapter proteins, have been shown to negatively regulate adhesion events in a variety of cell types. Dok2 has recently been shown to interact with integrin $\alpha_{IIb}\beta_3$ following platelet activation



and given its role in negatively regulating cell activation, Dok2 is a prime candidate for negatively regulating integrin $\alpha_{IIb}\beta_3$ in platelets.

In preliminary studies, using mice which are genetically deficient in Dok2, we have shown an important role for Dok2 in enhancing integrin $\alpha_{IIb}\beta_3$ adhesive function. This alteration in adhesive function is most pronounced under physiological blood flow conditions, such as those encountered in the normal circulation and as such represents a physiologically relevant phenomenon. In this project, we plan to identify the mechanisms by which Dok2 regulates platelet adhesion. This project will utilise a wide range of experimental techniques including confocal microscopy, western blotting, in vitro whole blood adhesion assays, in vivo models of thrombosis growth and intravital microscopy techniques, which will allow us to visualise the growth of thrombi in the circulation of a live mouse. Together these studies will establish if Dok2 is a suitable target for the development of a new class of anti-thrombotic compounds that may ultimately improve treatment for patients with cardiovascular disease.

Investigating a new anti-clotting approach: Regulation of platelet adhesion and thrombus formation by the GPIb/V/IX adhesion receptor

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Adhesion of circulating blood platelets to an injured blood vessel results in the formation of a blood clot. When this occurs in diseased vessels (eg. atherosclerosis), this normally protective cascade of events results in arterial thrombosis, which is responsible for heart attack and ischaemic stroke, which together, are the biggest cause of death in industrialised societies. One platelet receptor, the glycoprotein (GP) Ib/V/IX complex plays a major role in promoting both haemostasis and thrombosis. GPIb/V/IX has unique binding characteristics, allowing efficient platelet capture from the bloodstream, even under the most rapid blood flow conditions. This initial step in the haemostatic process is important in mediating subsequent platelet activation required for blood clot formation. Factors controlling the function of this receptor are therefore critical to the regulation of the normal clotting function of platelets as well as being potentially important in the development of thrombosis.

The GPIb/V/IX receptor is composed of 4 subunits; GPIb α , GPIb β , GPIX and GPV (see Figure 1). Current evidence demonstrates that the cytoplasmic tail of GPIb α plays an important role in platelet adhesion and thrombus formation through its interaction with cytoskeletal and signalling proteins. In recent studies we have developed a transgenic mouse expressing mutant forms of the GPIb α protein which no longer interact with filamin A. This project will involve characterisation of the platelets from these mice using a wide range of techniques including platelet functional assays, flow cytometry, confocal microscopy and in vitro models of thrombosis. These studies will establish the role of the GPIb α -filamin A interaction in platelet adhesion and thrombus formation which may represent a novel target for the development of new anti-thrombotic drugs.



A New Class of Antithrombotic Agents Investigating the role of PI 3-kinases in haemostasis and thrombosis

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Platelets play a key role in maintaining the integrity of the vasculature system by preventing excessive bleeding following traumatic injury and by providing the framework for vascular repair. Platelet adhesive function must be tightly regulated to avoid excessive platelet accumulation at sites of blood vessel injury, leading to pathological thrombus (clot) formation that causes heart attacks and stroke. A key intracellular signalling pathway controlling the adhesive function of platelets is the phosphatidylinositol 3-kinase (PI3K) pathway. This signalling pathway plays an important role in a range of human diseases including cancer, inflammation, autoimmune diseases and thrombosis. In a recent landmark study published in Nature Medicine our laboratory discovered a new class of antithrombotic agents that selectively inhibit a specific isoform of PI3K, termed PI3K p110beta. Inhibition of this enzyme represents a highly effective means of preventing pathological blood clots that appears safer and more effective than commonly used anticlotting agents.

In this project we will examine the effectiveness of PI3K p110beta inhibitors in preventing platelet activation and pathological blood clot formation in various animal stroke models. This study will be of interest to students aiming to learn about cell biology and signal transduction and in the processes underlying drug discovery and development. This project will use a broad range of experimental techniques, including flow-based adhesion assays, confocal microscopy, mouse models of carotid artery occlusion, histology, as well as a host of signal transduction and cell biology assays.

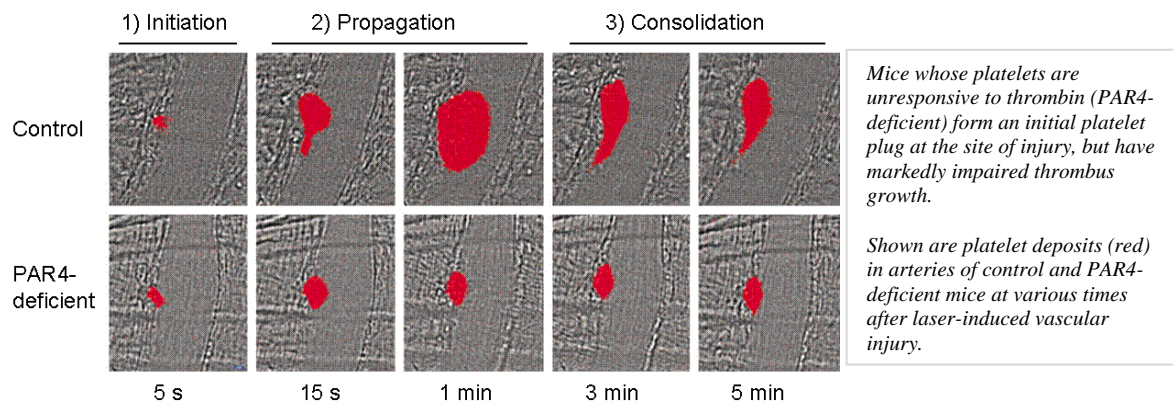
Defining the roles of platelet protease-activated receptors in thrombosis

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Platelets are essential components of arterial thrombi, which cause heart attacks and most strokes. Consequently, substantial research has been directed at defining the mechanisms by which platelets contribute to a growing thrombus since interfering with such mechanisms may afford effective antithrombotic approaches. This project will investigate the role of platelet thrombin receptors, termed protease-activated receptors (PARs), in thrombosis.

We have previously shown that mice which are genetically deficient in PARs are protected against experimental models of thrombosis yet do not exhibit spontaneous bleeding, indicating the potential of these receptors as targets for antithrombotic therapy in humans. This project will extend these studies to define the roles of PARs in thrombus growth and stability and to incorporate fundamental studies on the importance of PARs in human thrombus formation.

The project will utilise many useful techniques including mouse genetics, ex vivo whole blood flow experiments to examine clot formation, confocal microscopy, and in vivo mouse models of thrombosis and haemostasis. These studies will determine whether PARs are a suitable candidate for the development of anti-thrombotic therapies and will appeal to students interested in researching and developing future therapies targeting heart attacks and occlusive strokes.



Investigation of the functional interplay between platelets and neutrophils

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Platelets and neutrophils are very reactive cells in blood circulation in order to respond rapidly to vascular wall damage and inflammation, respectively. It has become clear that besides the fundamental role in haemostasis and thrombosis, platelets also regulate a variety of inflammatory responses and are key players in atherothrombosis. Thrombosis and inflammation is therefore linked rather than separate entities. As atherothrombotic diseases are a major cause of morbidity and mortality in developed countries, understanding the role of platelets in vascular inflammation and atherosclerosis is an important challenge.

Platelet adhesion to sites of vessel damage is inevitably accompanied with the release of granule content, protein surface expression and shedding, and formation of proinflammatory lipids. The platelet-neutrophil cross talk begins with the P-selectin expression which initiates signalling pathway in neutrophils to modulate integrin adhesiveness. The integrin activation is reinforced by the platelet derived chemokines to allow ligand engagement of the activated integrins to facilitate firm adhesion. The integrins are not only responsible for the attachment of neutrophils to platelets or endothelial cells but also able to transmit signals from the extracellular domain into the cell to strengthen adhesion, induce superoxide production, respiratory burst, granule release and transmigration to fulfil their defence function.

Despite the major achievements made over the years in the understanding of molecular mechanisms regulating platelet-neutrophil interaction, many questions still remain. For example, it is unclear how the various components of platelets such as the granule stored chemokines, P-selectin expression, inflammatory lipid production and microparticle formation, cooperatively regulate neutrophil activation. In addition, there is very limited information if platelet adherent neutrophils can in turn exert feedback effect to regulate platelet activation. With these questions in mind, this project will begin to address these issues by performing relevant studies to provide new insights into the underlying molecular mechanism governing the cross talk between platelets and neutrophils, and ultimately to help develop novel therapeutic strategies.



Fibrinolysis and Gene Regulation Unit

The role of mRNA stability in the regulation of tissue-type plasminogen activator gene expression

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Tissue-type plasminogen activator (t-PA) is a serine protease expressed in many cells including endothelial cells that forms an integral component of the plasminogen activating system. t-PA and a related protease, known as urokinase type plasminogen activator (u-PA) convert plasminogen into its active form, plasmin. Once formed, plasmin can degrade blood clots and also other proteins associated with the extracellular matrix. As such the plasminogen activators are linked with the invasive capability of tumour cells since metastatic cancers use plasmin to invade surrounding tissues and metastasize to distant locations. In addition to these effects, t-PA either alone or by generating plasmin, has been shown to have profound effects in the central nervous system, where it is linked to memory formation, synaptic plasticity and neurodegeneration. t-PA is expressed in many cells within the CNS, including neurons, yet the regulation of t-PA expression in this compartment is poorly understood. We are particularly interested in understanding how the expression of the t-PA gene is regulated in human fibroblasts (related to cancer metastasis), endothelial (related to blood clotting) and neuronal cell lines (related to CNS effects).

Regulation of gene expression can occur at many levels: transcription, post-transcription, translation, and protein secretion. Although the t-PA gene is regulated at all of these levels, the least studied is its regulation at the post-transcriptional and translational levels. In our laboratory, we have shown that t-PA mRNA contains sequences in the 3' untranslated region that can exert a powerful effect on the longevity of the mRNA itself (i.e. it alters t-PA mRNA stability), thereby establishing the importance of this level of control.

The Honours project available will focus initially on fibrosarcoma cells and neuronal cells and will more closely define the instability element(s) within the t-PA 5'- and 3' UTRs using *in vivo* RNA decay assays (standard procedure in the laboratory) and possibly *in vitro* RNA decay assays, and will also use RNA binding assays to determine if these regions provide binding sites for proteins. These studies will lay a foundation to identify these proteins *via* proteomic technologies. Moreover, this project will also assess any influences that the t-PA 5' UTR has on mRNA stability; and examine the possible role of the 3'UTR on the rate of translational initiation, as has previously been suggested in the literature. We will also be interested in comparing the relative contribution of post-transcriptional regulation to t-PA gene expression. This project will involve many aspects of molecular biology, cell culture (including the preparation of primary mouse embryonic neurons), RNA stability assays, RNA binding assays and biochemistry. This work will contribute to the broader, and increasingly important, field of post transcriptional gene expression.



Is the expression of the plasminogen activator inhibitor type 2 gene subject to Post-transcriptional regulation?

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Our laboratory is interested in understanding the mechanisms that govern gene expression of members the plasminogen activating (PA) system. The end product of this system is a powerful protease called plasmin that can degrade blood clots and a number of other proteins within the extracellular matrix. The generation of plasmin from plasminogen is mediated by the plasminogen activators: tissue-type plasminogen activator (t-PA) and urokinase (u-PA). The activity of the two plasminogen activators is regulated by specific inhibitors, known as PAI-1 (that inhibits t-PA and u-PA) and PAI-2 (that preferentially inhibits u-PA).

PAI-2 is an unusual protease inhibitor as the majority of PAI-2 is located intracellularly. In the last few years novel intracellular roles have been attributed to PAI-2, including as a survival gene in macrophages following bacterial infection. PAI-2 has also been shown to inhibit apoptosis and to modulate activity of a tumour suppressor protein. PAI-2 also enhances HIV replication while HIV infection increases PAI-2 expression. PAI-2 is also a highly regulated gene, with its level of expression being massively increased in cells under some conditions.

We have been studying the biology of PAI-2 with particular emphasis on gene regulation. We have shown that the fold induction of PAI-2 mRNA levels is dramatically stimulated by tumour promoting agents (e.g. phorbol esters) and cytokines (eg TNF α). Previous reports have suggested that the increase in PAI-2 mRNA is not only transcriptional, but also includes post-transcriptional events. Post transcriptional regulation is often controlled by sequences located in the 3' untranslated region (3'-UTR). To date we have mapped and identified three discrete sequence elements within a 120 nt region in the 3'-UTR of the PAI-2 transcript, and one coding sequence element within Exon 4, that markedly influence PAI-2 mRNA stability. Moreover, we have a number of candidate RNA binding proteins that could be involved in PAI-2 mRNA decay/stabilisation *via* the 3'UTR. The current hypothesis suggests that these 3' UTR-localised elements can modulate PAI-2 mRNA decay (e.g. either increase or decrease mRNA stability), and possibly even translational initiation, depending on the physiological status of the cell.

This Honours project will assess the contribution of these elements to the overall control of PAI-2 mRNA decay *in vivo* using human Fibrosarcoma (fibroblastic sarcoma) and U937 (myeloid leukaemia) model cell lines. This project will also use RNA binding assays to determine whether proteins selectively bind to one or more of these regions and if this is altered when cells are stimulated with agents [e.g. cytokines (TNF), carcinogens (phorbol ester) and glucocorticoids (dexamethasone)], that regulate PAI-2 gene expression. This work will provide a basis for a proteomic approach to identify these RNA binding proteins, and will not only add to the understanding of PAI-2 biology, but will also contribute to the broader, and increasingly important, field of post transcriptional gene expression.



ECRU Biotechnology Division

Histone Deacetylase Inhibitors as Novel Treatments for Vascular Disease

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The involvement of extracellular matrix (ECM) turnover in the progression of pathological vascular conditions such as vascular bypass graft failure due to neointimal hyperplasia (NI) and abdominal aortic aneurism (AAA) has been documented (Lijnen *Thromb Haemo* 86, 324-33, 2001).

The contribution of proteolytic enzyme cascades to increased ECM turnover in these conditions is implicated in disease progression. Inhibition of matrix degrading enzymes such as those of the plasminogen activating (PA) and matrix metalloproteinase (MMP) systems may be of benefit in the treatment of NI and AAA.

We have recently identified a series of novel compounds, acting as histone deacetylase inhibitors (HDACi) (Dear et al. *Organic and Biomolecular Chemistry* 21:3778-84 (2006), as able to inhibit MMP and u-PA activity (Cakarovski K et al. *Int.J.Cancer* 110:610-616 (2004). These agents have recently been demonstrated to reduce AAA formation in-vivo (Vinh A et al. *J.Vasc.Res* 45(2):143-52 2008).

As an extension of these basic and translational research activities undertaken in the laboratory, assessment of the therapeutic efficacy of novel HDACi (MCT-1 and MCT-2, 3 and 4), in the inhibition of NI progression is to be evaluated. Assessment of the efficacy of these agents will be undertaken in the apoE knock out mouse model provided by Associate Professor Rob Widdop, Department of Pharmacology, Monash University.

The project will involve determination of NI formation in an animal model, immunohistochemical characterisation and quantitative PCR analysis of u-PA, MMP and HDAC expression in plasma and tissue sections from control and treated animals and is likely to appeal to students interested in translational research.

Molecular interactions and assembly of platelet receptor signalling complexes in the initiation of thrombus formation

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The capacity of platelets to rapidly adhere to exposed vascular matrix at arterial and pathological shear rates is dependent on two adhesion receptor complexes unique to platelets, the glycoprotein (GP)VI/FcR γ -chain complex that binds collagen and the GPIb-IX-V complex that binds von Willebrand Factor (VWF). In recent years, it has become clear that reactive oxygen species (ROS) generated by NADPH oxidase play a role in regulating platelet function: increased oxidative stress promotes platelet aggregation, while antioxidants such as quercetin (found in onions, tea and wine) inhibit platelet aggregation.

The precise signaling pathways remain to be fully defined. The overall objective of this proposal is to define whether early signaling events involving GPVI/FcR γ -chain and GPIb-IX-V are redox regulated.

Experiments will involve measurement of intracellular and extracellular reactive oxygen species production by human platelets, assessment of temporal changes in platelet function following activation, and investigation of platelet function in NADPH oxidase-knockout mice.

Physiological and pathological significance of receptor shedding from platelets, and assessment of plasma soluble GPVI as a biomarker of thrombotic abnormalities

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Our Aim is to understanding the molecular mechanisms integral to platelet activation processes initiated by receptor/ligand engagement. In normal haemostasis and in situations leading to pathological thrombosis, platelet receptors orchestrate platelet activation in turn leading to platelet adhesion, aggregation and thrombus formation. The primary signalling receptors that trigger these molecular events include GPIb-IX-V and GPVI (the platelet receptors for von Willebrand factor and collagen respectively). Recently we have uncovered molecular mechanisms leading to shedding (removal) of these specific receptors from the platelet surface. By shedding these receptors, platelets can 'self-regulate' their ability to activate, and to adhere and spread on a blood vessel wall.

We have projects examining the role of receptor shedding in formation and dissolution of a thrombus. Clinically, excessive shedding of platelet receptors may indicate a prothrombotic or inflammatory situation and may indicate aberrant platelet activation leading to coronary vessel occlusion and stroke. We have designed assays to measure the extent of receptor shedding and will examine populations of people at risk of stroke and myocardial

infarction for plasma levels of shed receptor protein. The control of platelet receptor shedding also represents a novel target for therapeutic intervention. Project titles include:

1. How shedding of receptors from platelets exposed to shear force occurs.
2. Plasma levels of soluble GPVI as a clinical marker of platelet activation and inflammation and in patients at risk of stroke.

Identification of binding sites for GPIb-IX-V ligands, and how these interactions regulate cell adhesion and coagulation

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The unique platelet receptor of the leucine-rich repeat (LRR) family, glycoprotein (GP)Ib-IX-V, plays a central role in mediating platelet adhesion to the vessel wall at high shear stress, and controls procoagulant activity on activated platelets by binding thrombin, coagulation factors XI and XII, and kininogen. GPIb α (the major ligand-binding subunit of GPIb-IX-V) mediates adhesion of circulating platelets to subendothelial matrix by binding von Willebrand factor (VWF), or to intact endothelium by binding P-selectin on activated endothelial cells, or P-selectin-associated VWF multimers. The major ligand-binding domain of GPIb α is the extracellular N-terminal sequence His1-Glu282, consisting of seven LRR (Leu36-Ala200), N- and C-terminal flanking sequences (His1-Ile35 and Phe201-Gly268), and an anionic sequence Asp269-Glu282. The ligand-binding domain of GPIb α is conformationally-sensitive, and not amenable to analysis by short peptides or random scanning-mutagenesis. However, previous studies analysing cross-species human/canine chimeras of GPIb α expressed on CHO cells have mapped binding sites for VWF and inhibitory anti-GPIb α mAbs to specific structural regions. This approach is based on specificity of human VWF and murine mAbs for human (not canine) GPIb α , and identified LRR2-4 spanning an electronegative patch in Leu60-Glu128 as crucial for GPIb α -dependent adhesion to VWF.

The aims of this project are to expand these approaches to investigate binding to GPIb α of other ligands including procoagulant factors such as FXI or FXII, localized to platelets *via* binding to GPIb α . Deficiency of either FXI or FXII of the intrinsic coagulation pathway has a major impact on arterial thrombosis, but essentially no effect on normal haemostasis (bleeding time) in humans or mice. To analyse binding of these ligands to GPIb α , we have established a thrombinoscope-based assay for measuring platelet/GPIb α -dependent FXI/FXII mediated thrombin generation in human plasma, and have shown that selective depletion of GPIb α from human platelets (using the cobra metalloproteinase, Nk) inhibits FXII-dependent coagulation. This provides additional functional data supporting analysis of interactions between FXII and GPIb α as a potential anti-thrombotic target, and identified a new pathway for FXII activation in human plasma independent of normal contact activators.