Roles of coagulation factor XIII in the functions of blood platelets

Simbarashe G. Magwenzi

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ABSTRACT

Activated blood coagulation factor XIII (FXIIIa) is a transglutaminase that stabilises fibrin clots and associates with platelets. In the present study, the role of factor XIII (FXIII) in modulating physiological platelet functional responses including adhesion, signal transduction and spreading were examined. Under static conditions, platelets adhered to surface-immobilised plasma-purified and recombinant human FXIII leading to the formation of filopodia and lamellipodia. Adhesion to FXIIIa was mediated through integrin-dependent mechanisms, since it was abolished by treatment with RGDS. Moreover, platelet adhesion to FXIIIa was reduced partially, but significantly by either the specific integrin $\alpha_{IIb}\beta_3$ antagonist tirofiban or the selective $\alpha_v\beta_3$-blocking antibody LM609, and abolished when used in combination. However, spreading was exclusively mediated by $\alpha_{IIb}\beta_3$ since it was ablated by tirofiban, but unaffected by LM609. Importantly, FXIIIa-mediated platelet accrual was preserved under venous and arterial flow conditions where both integrins played essential roles. Under these conditions, platelet adhesion to immobilised activated FXIII (FXIIIa) was apparent at a shear rate of $300s^{-1}$, significantly reduced at $800s^{-1}$, but absent above $1000s^{-1}$. These platelet-FXIII interactions occurred independently of FXIII transglutaminase and protein disulfide isomerase activities. However, platelet adhesion and spreading were abolished by the Src family inhibitor PP1 indicating a tyrosine kinase-dependent mechanism. Consistent with this, FXIIIa stimulated tyrosine-phosphorylation of several proteins including Syk, SLP-76 and PLCγ2 but not LAT, in adherent platelets. FXIIIa immobilised rapidly on collagen, and enhanced collagen-induced thrombus formation at a shear rate of $800s^{-1}$. When co-

immobilised with fibrinogen and vWF, the coagulation factor also accentuated platelet accrual by these key platelet adhesive proteins also at arterial shear. These data provide evidence that FXIIIa supports platelet adhesion under flow and potentiates the thrombogenic effects of established platelet ligands, suggesting a novel role for FXIIIa in enhancing platelet-dependent haemostasis.

PUBLICATIONS

Published Articles

Magwenzi, S.G., Ajjan, R.A., Standeven, K.F., Parapia, L.A., & Naseem, K.M. (2011). Factor XIII supports platelet activation and enhances thrombus formation by matrix proteins under flow conditions. *Journal of Thrombosis and Haemostasis*, 9: 820-833 {Impact factor 6.3}

Roberts, W., Magwenzi, S.G., Aburima, A.A., Naseem, & K.M. (2010). Thrombinspondin-1 induces platelet activation through CD36-dependent inhibition of the cAMP/protein kinase A signalling cascade. *Blood*, 116: 4297-4306 {Impact factor 10.5}

Oral Presentations

A new paradigm in factor XIII-platelet interactions under static and flow conditions: the role of integrin receptors. *Northern Cardiovascular Research Group (NCRG) meeting, Manchester UK* (2010)

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Poster Presentations

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Magwenzi, S.G., Parapia, L.A. & Naseem, K.M. Activated factor XIII supports platelet adhesion under static and flow conditions through an integrin-mediated mechanism. *10th UK platelet meeting, Bradford UK* (2008)

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LIST OF ABBREVIATIONS

$\alpha_2\beta_1$	Integrin alpha 2 beta 1
$\alpha_{IIb}\beta_3$	Integrin alpha IIb beta 3
$\alpha_v\beta_3$	Integrin alpha v beta 3
Ab	Antibody
jACD	Acid citrate dextrose
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ANOVA	Analysis of variance
APS	Ammonium persulphate
ATP	Adenosine 5'-triphosphate
BAPTA-AM	1,2-bis-(o-Aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid, tetra(acetoxymethyl)ester
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine 3',5'-monophosphate
CCD	Charge-coupled device
cFXIII	Cytoplasmic factor XIII
cGMP	Cyclic guanosine 3',5'-monophosphate
COX-1	Cyclo-oxygenase 1
DAG	Diacylglycerol
$DiOC_6$	3,3'-dihexyloxacarbocyanine iodide
DMSO	Dimethyl sulphoxide
DTS	Dense tubular system
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid

FcRγ	Fc receptor gamma-chain
FITC	Fluorescein isothiocyanate
FXIII	Coagulation factor XIII, also used to specify the inactive enzyme by convention
FXIII-A	A-subunit of factor XIII
FXIIIa	Enzymatically active form of factor XIII
FXIII-B	B-subunit of factor XIII
GP	Glycoprotein
GP1b-IX-V	Glycoprotein 1b-IX-V receptor complex
GPIaIIa	Glycoprotein IaIIa, also called $\alpha_2\beta_1$
GPIIbIIIa	Glycoprotein IIbIIIa, also called $\alpha_{IIb}\beta_3$
GPCR	G-protein coupled receptor
GPVI	Glycoprotein VI
GSNO	S-nitrosoglutathione
GT	Glanzmann's Thrombasthenia
GTP	Guanosine 5'-triphosphate
HRP	Horseradish peroxidise
IB	Immunoblot
IgG	Immunoglobulin G
IP	Immunoprecipitate
ITAM	Immunoreceptor tyrosine-based activation motif
LAT	Linker for activation of T cells
LIBS	Ligand induced binding site
LM609	Mouse anti-integrin alpha v beta 3 antibody
Mg^{2+}	Magnesium ion
MI	Myocardial infarction
MIDAS	Metal ion-dependent adhesion site
NIH	National Institutes of Health
NO	Nitric oxide
OCS	Open canalicular system
PAR (1 and 4)	Protease activated receptor (1 and 4)

PBS	Phosphate- buffered saline
PDI	Protein disulphide isomerase
PGE_1	Prostaglandin E_1
PGI_2	Prostaglandin I_2, also called prostacylin
PH	Pleckstrin homology
PI 3-kinase	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLCβ	Phospholipase C beta
PLCγ2	Phospholipase C gamma 2
PRP	Platelet rich plasma
PVD	Peripheral vascular disease
PVDF	Polyvinylidene fluoride
pTyr	Phosphotyrosine
RBC	Red blood cells
RGDS	Arginine-glycine-aspartic acid-serine
rFXIII	recombinant factor XIII, also used to specify the inactive recombinant enzyme by convention
rFXIII-A	recombinant A-subunit of factor XIII
rFXIIIa	Enzymatically active recombinant factor XIII
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sGC	Soluble guanylyl cyclase
SH2	Src Homology 2 domain
SLP-76	Src homology 2 domain-containing leukocyte protein of 76 kDa
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline- tween
TEMED	Tetramethylethylenediamine

TRITC	Tetramethyl Rhodamine Iso-Thiocyanate
TXA_2	Thromboxane A_2
vWF	von Willebrand factor

Amino acid abbreviations

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic Acid
E	Glu	Glutamic Acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

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LIST OF ACCOMPANYING MATERIAL

Accompanying this thesis is a compact disc with the following videos:

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Video 2: Platelets adhere to FXIIIa at an arterial shear of $800s^{-1}$

Video 3: Platelet adhesion to FXIIIa is observable in flowing whole blood at $300s^{-1}$

Video 4: Inhibition of $\alpha_{IIb}\beta_3$ with tirofiban reduces platelet adhesion to FXIIIa under flow at $300s^{-1}$

Video 5: Inhibition of $\alpha_v\beta_3$ with LM609 reduces platelet adhesion to FXIIIa under flow at $300s^{-1}$

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Simba

AUTHOR'S DECLARATION

I confirm that this work is original and that if any passage(s) or diagram(s) have been copied from academic papers, books, the internet or any other sources these are clearly identified by the use of quotation marks and the reference(s) is fully cited. I certify that, other than where indicated, this is my own work and does not breach the regulations of HYMS, the University of Hull or the University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS Code of Practice on Academic Misconduct, and state that this piece of work is my own and does not contain any unacknowledged work from any other sources.

CHAPTER 1 - GENERAL INTRODUCTION

1.1 Introduction

Haemostasis is initiated following blood vessel injury to preclude loss of blood. It involves concerted actions of blood platelets and soluble coagulation factors in forming blood clots at sites of vascular injury. Platelets are recruited from flowing blood and become activated to fulfil their haemostatic roles (Marcus, 1969, Ruggeri and Mendolicchio, 2007). Coagulation factors are also spatially activated at these sites where they initiate, promote or reinforce platelet activity (Heemskerk et al., 2002). Interactions between these two elements of the haemostatic machinery enhance clot formation and stabilisation. Although platelets provide an essential defence against life-threatening haemorrhage, their superfluous or unregulated activation can also lead to pathological outcomes (Ruggeri, 2002). For instance, chronic vascular damage sustained in atherosclerosis results in platelet-mediated vascular occlusion (Ross, 1986). The consequent restriction of blood supply to organs and tissues results in ischemia, which leads to myocardial infarction (MI) and stroke when the heart and brain are affected respectively. This chapter will review literature forming the basis of current understanding of platelet biology, coagulation factor XIII (FXIII) function and known interactions between the two.

1.2 Platelets

Platelets are the smallest blood cells and they are crucial for maintaining vascular integrity. Their continuous synthesis and replenishment ensures constant availability in blood, while their structure is well-adapted for haemostatic roles.

1.2.1 Platelet synthesis

Pluripotent stem cells in the bone marrow, liver and kidney differentiate into megakaryocytes which release platelets through mechanisms critically dependent on thrombopoeitin (Kelemen et al., 1958, Lok et al., 1994, de Sauvage et al., 1994). Mature megakaryocytes extend their peripheral membranes into long projections with terminal ends swollen into structures called proplatelets (Italiano et al., 1999). Proplatelets endowed with organelles from parent megakaryocyte cytoplasm eventually bud off to enter the circulation as platelets (Becker and De Bruyn, 1976). A single megakaryocyte gives rise to 1000 to 3000 platelets and approximately 1×10^{11} new platelets are produced each day; maintaining normal human blood platelet concentration at 150×10^{9} - 400×10^{9} platelets/L (Kaushansky, 2005). Platelet lifespan is around 10 days and senescent platelets are depleted from blood by the reticuloendothelial system in the spleen (Cohen and Leeksma, 1956, Aas and Gardner, 1958, Aster, 1966). About a third of the body's platelet pool is normally sequestered in the spleen and platelets are released from this storage site to meet situations of increased demand (Aster, 1966).

1.2.2 Platelet ultrastructure

Platelet ultrastructure has become well-defined from observations made under electron microscopy (White, 1979). Human platelets are 2-5µm in diameter and are approximately 0.5µm thick with a mean cell volume of 6 to 10 femtolitres. Under resting conditions, platelets are biconvex discs. However, their shapes can change dramatically following activation (Figure 1.1).

1.2.2.1 Platelet membranes and cytoskeleton

Platelets are surrounded by a plasma membrane which has numerous infolding crevices that give it an appearance resembling sulci and gyri on the brain surface (White et al., 1995). Similar to other cells, the platelet plasma membrane is a phospholipid bilayer comprising the phospholipids: phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine (Marcus, 1978). An important feature is the presence of multiple invaginations which form a convoluted network of channels through the cytoplasm called the open canalicular system (OCS) (White and Clawson, 1980). This increases the platelet surface available for interaction with plasma and enhances absorption and release of substances. Moreover, the OCS provides extra membrane required for platelet spreading (White and Clawson, 1980, White, 2005). In addition to the OCS, platelets also possess a separate channel network called the dense tubular system (DTS); a remnant of megakaryocyte smooth endoplasmic reticulum which serves as a calcium storage site (White, 2002).

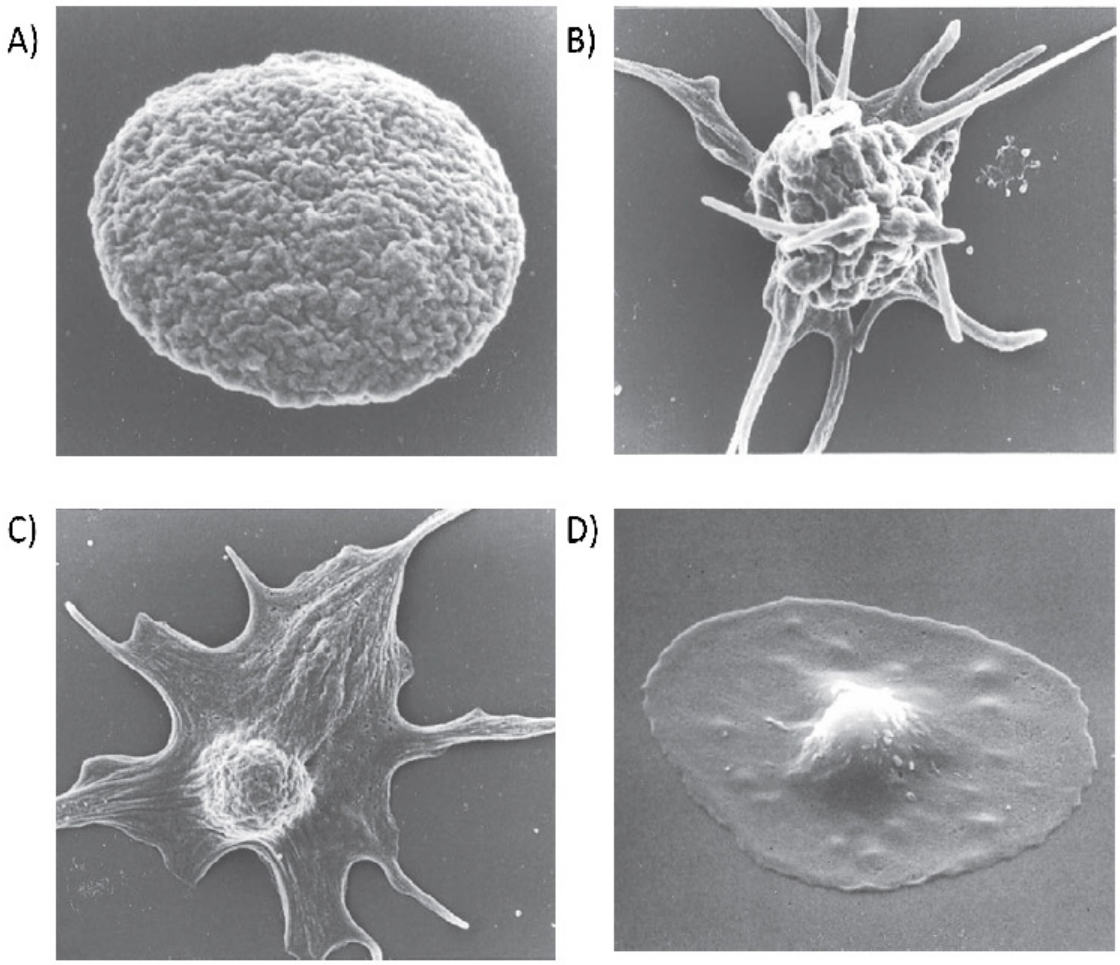


Figure 1.1: Platelet ultrastructure. **(A)** Discoid platelet photographed in the low-voltage, high-resolution scanning electron microscope (LVHR-SEM). The outside of the cell has a wrinkled appearance resembling gyri and sulci on the surface of the brain. Magnification ×30,000. **(B)** Platelet in early activation viewed by LVHR-SEM. Fine processes (filopodia) extend in all directions. Magnification ×13,000. **(C)** Early spread platelet with observable lamellipodia. The central body of the cell remains convoluted, but is gradually disappearing as the cytoplasm spreads and fills the spaces between filopodia. Magnification ×11,000. **(D)** Spread platelet viewed by conventional SEM. Magnification ×9,000. Figure and legend are compiled from figures in reference (White, 2002).

Release of calcium from this store into the cytoplasm drives a number of platelet activation responses. The DTS is also the site of platelet cyclo-oxygenase 1 (COX-1) which plays a critical role in the generation of thromboxane A_2 (TXA_2); a major contributor to platelet recruitment at sites of thrombus formation (Gerrard et al., 1978, Carey et al., 1982).

Immediately beneath the platelet membrane is a framework of proteins that form the platelet cytoskeleton. The organisation of the cytoskeleton controls resting platelet structure and also drives morphological changes that accompany platelet activation (Escolar et al., 1986). The cytoskeleton comprises of microtubules, actin filaments, spectrin strands and other associated proteins. Some platelet surface receptors such as integrins and GP1b are attached to it at their cytoplasmic tails; thus mediating firm linkage between the cytoskeleton and externally bound ligands (Hartwig and DeSisto, 1991, Shattil and Newman, 2004). The discoid platelet appearance is maintained by a single microtubule which coils around itself about 9 times along the wider edge of a platelet (White, 2002). Following platelet stimulation, globular actin polymerises into filaments in an energy-dependent manner that utilises cytoplasmic ATP (Jennings et al., 1981). Actin filaments facilitate platelet shape changes by pressing outwards against the inner face of the membrane. Through interactions with myosin, actin also takes part in several contractile responses involved in stabilising platelet thrombi (Shepro et al., 1970, Pollard, 1980)

1.2.2.2 Platelet receptors

The external platelet membrane surface is covered a myriad of glycoprotein (GP) receptors which form a prominent coat called the glycocalyx (Kieffer and Phillips, 1990). The receptors

present on the platelet surface perform diverse functions including adhesion to exogenous ligands leading to signal transduction which either activates or inhibits platelet haemostatic function. Platelet surface receptors can be divided into several receptor families including; integrins, G-protein-coupled receptors (GPCR), tyrosine kinase receptors, tetraspanins, receptors belonging to the immunoglobulin superfamily, the C-type lectin family and leucin rich-repeat family (Kieffer and Phillips, 1990, Rivera et al., 2009). Noteworthy among platelet receptors are various GPCRs including those for TXA_2 and adenosine 5'-diphosphate (ADP), collagen receptors GPIV and GPIaIIa (integrin $\alpha_2\beta1$), the von Willebrand factor (vWF) receptor complex GP1b-IX-V, and the fibrinogen-binding receptor GPIIbIIIa (integrin $\alpha_{IIb}\beta_3$) which are critical for platelet thrombus formation. There are approximately 80,000 copies of $\alpha_{IIb}\beta_3$ on the platelet surface, making it the most highly expressed surface receptor (Wagner et al., 1996).

1.2.2.3 Platelet organelles and granules

Similar to other cells, platelet cytoplasm contains different organelles, some of which play important roles during thrombus formation. Like erythrocytes, platelets distinctly lack a nucleus, which limits their proteome to proteins synthesised by their parent megakaryocytes. However, platelets retain residual mRNA from megakaryocytes which may enable them to synthesise new proteins when activated such as the proinflammatory cytokine interleukin 1β (Lindemann et al., 2001a, Lindemann et al., 2001b).

Platelet cytoplasm has a diverse granular content including alpha (α) granules, dense (δ) granules and lysosomes. α-granules are the most abundant and number 40-80 per platelet

(White, 2002). They are 0.2-0.4µm in diameter and are identifiable under electron microscopy by densely staining cores (White, 2002). They contain various pro-thrombotic mediators which are secreted into the OCS following platelet activation. These include vWF, fibrinogen, thrombospondin, vitronectin, platelet derived growth factor (PDGF), platelet factor 4 (PF_4), β-thromboglobulin, factor V and FXIII (Marx et al., 1993, White, 2002). P-selectin and $\alpha_{IIb}\beta_3$ integrins are present on the membranes of alpha granules and become exposed on the platelet surface following fusion of α-granule membranes with the external platelet membrane. By comparison, δ-granules are smaller than α-granules, appear more densely stained under electron microscopy and are less abundant. They are about 0.15µm in diameter and typically 3-8 δ-granules are found per platelet (McNicol and Israels, 1999, White, 2002). δ-granules also secrete their contents during platelet activation. These include ADP, adenosine 5'-triphosphate (ATP), calcium ions (Ca^{2+}), pyrophosphates and serotonin (5-HT). Platelet lysosomes maintain an internal acidic pH and contain hydrolytic enzymes. A few glycogen granules are also present within platelets which provide an energy store since glycogen can be metabolised into glucose. Platelets are able to carry out both anaerobic and aerobic respiration, with the latter being facilitated by mitochondria which are also present in the platelet cytoplasm (Kitchens and Newcomb, 1968).

1.2.3 The roles of platelets in haemostasis

Platelets have been described as "sentinels of vascular integrity, [that] adhere where alterations are detected, and signal the abnormality to other platelets and blood cells" (Ruggeri, 2008). This essentially summarises their haemostatic roles since they permeate the vasculature in quiescent states with their spatial activation being restricted to sites of vascular injury. A continuous layer of endothelium lines the lumens of blood vessels and provides an inert surface that is normally refractory to platelet adhesion. Due to collisions with larger red blood cells (RBC) in flowing blood, platelets are forced away from the centre of vascular lumens towards the endothelium through a process termed *margination* (Aarts et al., 1988, Hathcock, 2006). This well positions them to detect changes in the structure and continuity of the endothelium. When such changes are detected, platelets are activated in stages which have been conceptually divided into initiation, extension and perpetuation phases (Figure 1.2) (Brass, 2003).

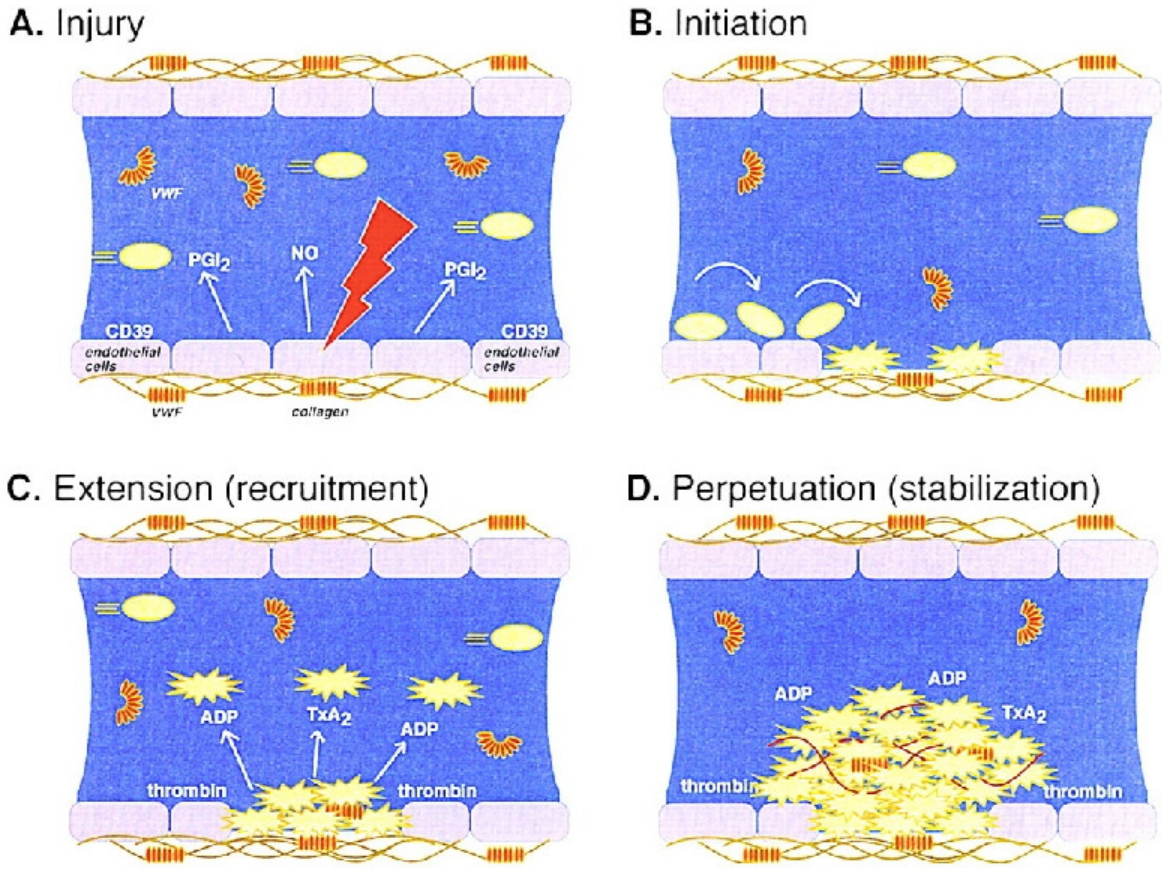


Figure 1.2: Stages of platelet plug formation. (A) Prior to vascular injury, platelets are restrained from activation by inhibitory factors that include prostacyclin (PGI_2) and nitric oxide (NO) released from endothelial cells, the presence of the ectonucleotidase CD39 on the surface of endothelial cells, and the inability of normal plasma von Willebrand factor (VWF) to bind spontaneously to the platelet surface. **(B)** The development of the platelet plug can be initiated by the exposure of collagen and VWF in the vessel wall. Rolling platelets adhere and spread on the collagen matrix, forming a monolayer of activated platelets. **(C)** The local generation of thrombin occurs rapidly on the surface of activated platelets and together with secreted adenosine diphosphate (ADP), and Thromboxane A_2 (TxA_2) facilitates the recruitment of flowing platelets. **(D)** During the perpetuation stage, close contacts between platelets promote the growth and stabilization of the haemostatic plug, in part through contact-dependent signalling mechanisms. Figure and legend are reproduced in modified form from reference (Brass, 2003).

1.2.3.1 Initiation phase

The initiation phase is triggered by discontinuation in the endothelium caused by vascular injury which exposes platelets to various adhesive subendothelial ligands (Figure 1.2B). These include collagen, fibronectin, thrombospondin and vWF (Ruggeri and Mendolicchio, 2007). Collagen in particular is the major subendothelial platelet adhesive protein which recruits flowing platelets to injury sites where they adhere and form a primary monolayer (Baumgartner, 1977, Nieswandt and Watson, 2003). However, under high shear (above a threshold of 800s^{-1}) typically found in the arterial circulation, platelets require vWF to enable their interactions with collagen (Weiss et al., 1978a). vWF is a multimeric protein produced by endothelial cells with a presence in the subendothelium, platelet α-granules and plasma. Plasma vWF can localise on exposed collagen fibres where its conformation is changed to one that allows rapid interactions with platelet GP1b/IX/V receptor complexes (Ruggeri and Ware, 1993). Although transient, platelet-vWF interactions retard platelet flow velocity, enabling the occurrence of less rapid receptor-ligand interactions such as those with collagen (Ruggeri and Mendolicchio, 2007).

The platelet receptors facilitating adhesion to collagen are $\alpha_2\beta_1$ integrins and GPVI (Farndale et al., 2004). When bound to GPVI, collagen stimulates biochemical signalling events that lead to platelet activation. Collagen cross-links GPVI resulting in tyrosine phosphorylation of Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) on Fc receptor γ-chains (FcRγ) which are co-expressed and physically associated with GPVI on the platelet surface (Gibbins et al., 1997, Poole et al., 1997, Tsuji et al., 1997). These phosphorylation events are mediated

by Src-family kinases, namely Lyn and Fyn and lead to the recruitment of the tyrosine-kinase Syk to phosphorylated FcRγ ITAMs where it becomes activated by autophosphorylation (Ezumi et al., 1998, Suzuki-Inoue et al., 2002, Watson et al., 2005). Activated Syk phosphorylates multiple targets including the adaptor proteins; linker for activation of T-cells (LAT) and SH2 domain containing leukocyte protein of 76 kilodaltons (SLP-76) (Watson et al., 2001). This leads to the assembly of a signalosome that results in the phosphorylation and activation of the gamma2 isoform of phospholipase C (PLCγ2) (Daniel et al., 1994, Asselin et al., 1997) and phosphatidylinositol 3-kinase (PI 3-K) (Pasquet et al., 1999). PLCγ2 hydrolyses membrane phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) into diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3). DAG activates the serine-threonine kinase protein kinase C (PKC) while IP_3 binds to its receptors on the DTS leading to calcium release into the cytoplasm. While PKC activation and intracellular calcium mobilisation can each initiate platelet shape change, spreading, secretion and aggregation, full platelet activation occurs through synergism between the two mediators (Kaibuchi et al., 1983). PI 3-K phosphorylates phosphoinositides in the membrane on the third position hydroxyl group of phosphatidylinositol (PI) thus transforming $PI(4,5)P_2$ and PI(4)P to $PI(3,4,5)P_3$ and $PI(3,4)P_2$ respectively. This facilitates pleckstrin homology (PH)-domain mediated membrane recruitment of PLCγ2 and Bruton's tyrosine kinase; a Tec kinase implicated in PLCγ2 activation (Kaibuchi et al., 1983).

Primary adherent platelets undergo activatory morphological changes following stimulation (Allen et al., 1979). An early change is the disintegration of the microtubule coil which transforms discoid platelets into spheres. Actin filaments are then cleaved by gelsolin which

is activated by elevated intracellular calcium (Lind et al., 1982). New actin polymerisation beneath the membrane then causes extension of finger-like projections called filopodia. Membrane segments between filopodia gradually coalesce, forming planar regions called lamellipodia and eventually platelets attain a fully spread morphology (Figure 1.1). Platelet spreading increases the surface area covered by each platelet and facilitates stable adhesion that resists shear forces imposed by flowing blood.

1.2.3.2 Extension phase

Once a primary adherent monolayer of platelets has been deposited, the extension phase follows (Figure 1.2C). This involves capture of free-flowing platelets from the blood by adherent platelets to form thrombi, a process termed platelet aggregation (Born, 1962, Jackson, 2007).

Activated adherent platelets release ADP and TXA_2, secondary agonists which act locally to activate proximal platelets. ADP is released by degranulation of platelet δ-granules which associate with the plasma membrane to empty their contents. Platelets have two purinergic receptors for ADP on their surfaces; $P2Y_1$ and $P2Y_{12}$. $P2Y_1$ is coupled to $G\alpha_q$ which activates the beta isoform of phospholipase C (PLCβ). Similarly to the mechanism described for PLCγ2 (section 1.2.3.1), PLCβ triggers calcium mobilisation and PKC activation. $P2Y_1$ also signals through $G\alpha_{12/13}$ (Jin et al., 1998). $P2Y_{12}$, is coupled to $G\alpha_i$ which inhibits adenylate cyclase thereby decreasing platelet intracellular cyclic adenosine 5'-monophosphate (cAMP) levels (Jin and Kunapuli, 1998). Since cAMP triggers a platelet inhibitory cascade, ADP signalling through $P2Y_{12}$ potentiates platelet activation by several agonists (Herbert and Savi, 2003).

Intracellular calcium mobilisation activates cytoplasmic phospholipase A (PLA) which cleaves phosphatidylcholine in the platelet membrane, liberating arachidonic acid. Following metabolic changes involving COX-1 and thromboxane synthase, arachidonic acid is converted to TXA_2. TXA_2 then diffuses out of the platelet where it binds TPα and TPβ receptors which are both linked to $G\alpha_q$ and $G\alpha_{12/13}$ (Shen and Tai, 1998, Klages et al., 1999).

While ADP and TXA_2 are able to initiate platelet aggregation, the most potent physiological agonist for this process and hence the extension phase is thrombin (Davey and Luscher, 1967). Following activation by collagen, platelets in the monolayer express negatively charged phosphatidylserine on their external surfaces by membrane ruffling which provides an ideal surface for the capture of soluble coagulation factors and initiation of the coagulation cascade that leads to thrombin generation (Lindhout et al., 1982, Bevers et al., 1985, Solum, 1999). Thrombin is a serine protease that cleaves terminal ends of its platelet G-protein coupled receptors which then rejoin as tethered ligands (Vu et al., 1991). Thrombin-mediated platelet activation occurs through protease activated receptors 1 and 4 (PAR1 and PAR4) which are linked to the $G\alpha_q$ G-protein subtype and activate PLCβ (Vu et al., 1991, Xu et al., 1998, Kahn et al., 1999). PAR1 and PAR4 are also linked to the $G\alpha_{12/13}$ G-protein subtype that triggers platelet shape changes by facilitating myosin interactions with actin in a Rho-A kinase dependent manner (Offermanns et al., 1994, Klages et al., 1999).

As described for ADP, TXA_2 and thrombin, the major platelet agonists mediating the extension phase bind to GPCR's (Figure 1.3). Other mediators signalling through GPCR's include 5-HT and epinephrine. However, their contributions to the extension phase are comparatively modest. Through ensuing signalling events, these agonists transform platelet surface integrins, particularly $\alpha_{IIb}\beta_3$, from low affinity conformations in resting platelets to the high affinity conformations found in activated platelets. The intracellular biochemical protein interactions stimulated by receptor ligation which lead to $\alpha_{IIb}\beta_3$ activation are collectively termed "inside-out signalling" (Ginsberg et al., 1992). Platelets with activated $\alpha_{IIb}\beta_3$ integrins aggregate by binding divalent plasma fibrinogen and multivalent vWF which bridge associations between platelets in thrombi.

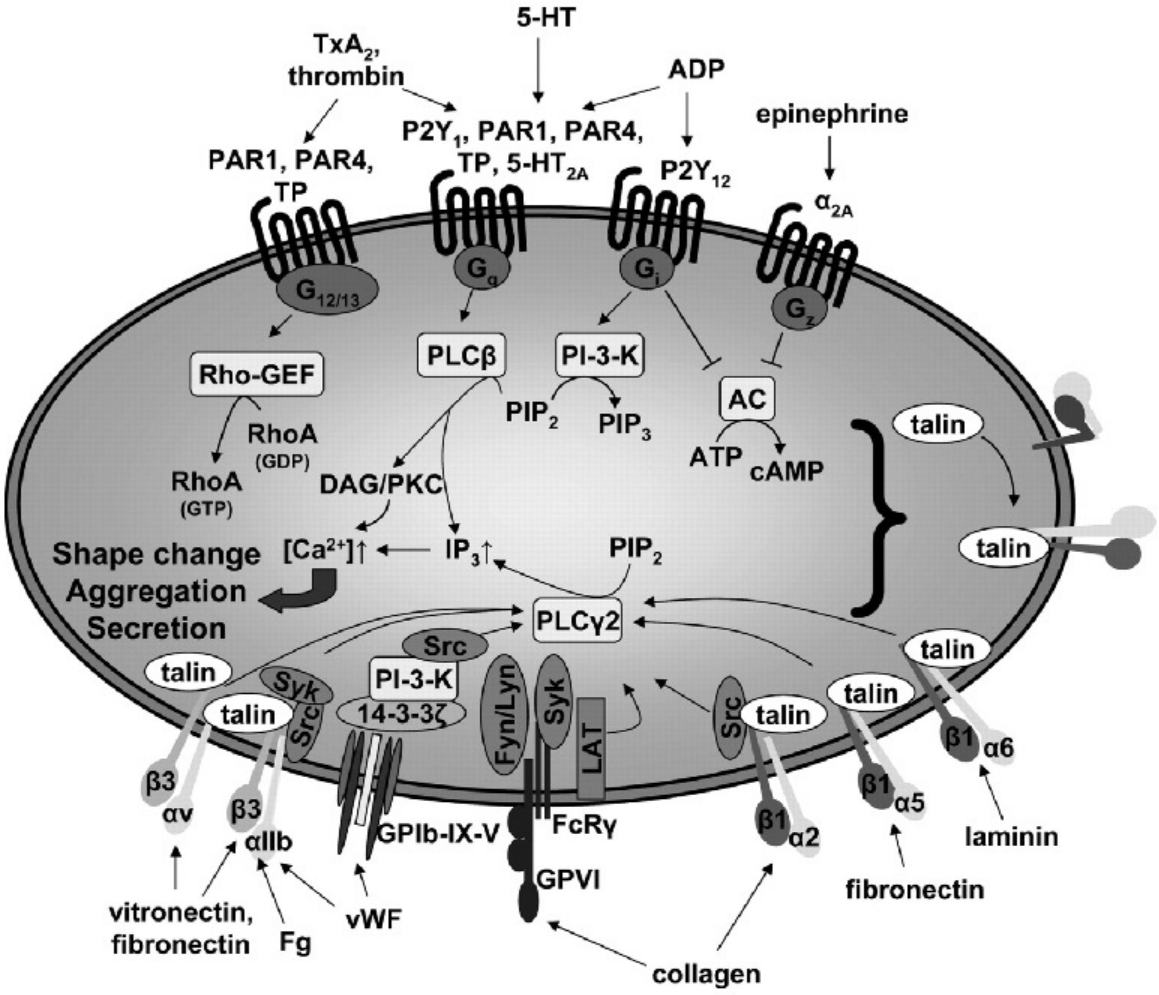


Figure 1.3: Signalling mechanisms linking platelet receptors to integrin activation. GPVI ligation activates the ITAM-signalling pathway, whereas stimulation of G protein–coupled receptors triggers pathways involving $G\alpha_q$, $G\alpha_{i/z}$, and $G_{12/13}$ and adenylate cyclase. DAG indicates diacylglycerol; IP_3, inositol-1,4,5-trisphosphate; PI-3-Kβ/γ, phosphoinositide-3-kinase β/γ; PIP_2, phosphatidylinositol-4,5-bisphosphate; PIP_3, phosphatidylinositol-3,4,5-trisphos-phate; PKC, protein kinase C; PLCβ2/3, phospholipase C-β2/3; RhoGEF, Rho-specific guanine nucleotide exchange factor. Figure and legend are reproduced in modified form from reference (Varga-Szabo et al., 2008).

1.2.3.3 Perpetuation phase

Once platelets have been recruited into thrombi, the perpetuation phase ensures long-term stability of adhesive contacts (Figure 1.2D). The most well understood mechanism operating to that effect is triggered by fibrinogen binding to $\alpha_{IIb}\beta_3$ which stimulates "outside in" signal transduction (Phillips et al., 2001). This perpetuates intracellular calcium mobilisation, PKC and PI 3-K activation thus maintaining high integrin ligand-affinity (Shattil et al., 1998). It also causes clot retraction which in addition to strengthening thrombi, reduces thrombus volume, thus limiting obstruction to vascular blood flow (Cohen et al., 1982, Shattil et al., 1998). Recent advances have revealed other mediators involved in thrombus stability including ephrins and platelet surface ephrin kinase receptors (Prevost et al., 2002), growth arrest specific gene 6 (Gas-6) (Angelillo-Scherrer et al., 2005), CD40 (Andre et al., 2002) and semaphorin 4D (Zhu et al., 2007). Clot stability ensures successful haemostasis, prevents embolism which may cause fatal vascular occlusion and localises platelets at sites where their secretion of various growth factors facilitates wound healing.

1.2.4 Platelet integrin receptors

Integrins are type I membrane receptors with an intracellular C-terminal, a single transmembrane domain and an extracellular N-terminal that facilitate cell-cell and cell-ligand contacts. They are heterodimeric glycoproteins consisting of α and β chains. To date, 18α and 8β chains have been identified on various cell types and these complex in different combinations giving 24 known integrins (Shattil et al., 2010). Platelets express 6 integrins including $\alpha_{IIb}\beta_3$ which binds fibrinogen, $\alpha_v\beta_3$ (vitronectin), $\alpha_2\beta_1$ (collagen), $\alpha_5\beta_1$ (fibronectin),

$\alpha_6\beta_1$ (laminin) and $\alpha_L\beta_2$ which has poorly defined ligand specificity. Through attempts to identify ligands selectively bound by each integrin, it emerged that many integrins are promiscuous and bind multiple ligands (Ruoslahti and Pierschbacher, 1987). For example, although $\alpha_{IIb}\beta_3$ is commonly referred to as the "platelet fibrinogen receptor" it also binds vWF, vitronectin and fibronectin (Phillips et al., 1988). Broad integrin ligand-specificity is caused by the binding of specific amino acid sequences found on several different proteins. Platelet $\alpha_v\beta_3$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ integrins bind their ligands through an arginine-glycine-serine (RGD) sequence while $\alpha_2\beta_1$ binds collagen at glycine-phenylalanine-hydroxyproline-glycine-glytamic acid-arginine (GFOGER) sequences (Humphries et al., 2006). Although $\alpha_{IIb}\beta_3$ binds vWF, thrombospondin, vitronectin and fibronectin through RGD, it has been demonstrated that physiological fibrinogen binding by the integrin is critically dependent on a lysine-glutamine-alanine-glycine-aspartic acid-valine (KQAGDV) sequence present on fibrinogen γ-chains (Kloczewiak et al., 1982, Kloczewiak et al., 1983). This occurs despite the presence of two RGD sequences on fibrinogen Aα-chains which play less important roles in platelet haemostasis (Zaidi et al., 1996).

1.2.4.1 Integrin structure

The crystal structures of the two platelet β_3 integrins ($\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$) have been solved (Xiong et al., 2001, Xiong et al., 2002, Xiao et al., 2004). From their membrane insertions to N-terminals, the extracellular regions of α_{IIb} and α_v are divided into calf domains, thigh domains and β-propellers consisting of repeated amino acid sequences arranged into 7 blades. The β_3 chain consists of a β tail domain, cysteine-rich EGF repeats with 3 disulphide bonds, a hybrid domain, a β-A domain and a Plexin-Semaphorin-Integrin (PSI) domain that

forms an inverted N-terminus (Figure 1.4). All three chains have "genu" which are kinks along their lengths that give $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ bent conformations associated with low-affinity resting states. Ligand binding is mediated by β-propellers in the α-chains and the β-A domain of the β_3 chain which together appear like a "head" supported by two "stalks" under electron microscopy. Several divalent cation sites are present on α and β chains that are important for tertiary structure and for ligand binding. The β_3 chain in particular has 3 metal ion binding sites that are involved in ligand binding: a metal ion dependent adhesion site (MIDAS) that prefers Mg^{2+}/Mn^{2+} which bind aspartic acid in RGD sequences, an adjacent to MIDAS sequence (ADMIDAS) whose cation regulates the interaction and a ligand induced metal binding site (LIMBS) whose cation stabilises ligand binding (Xiong et al., 2003).

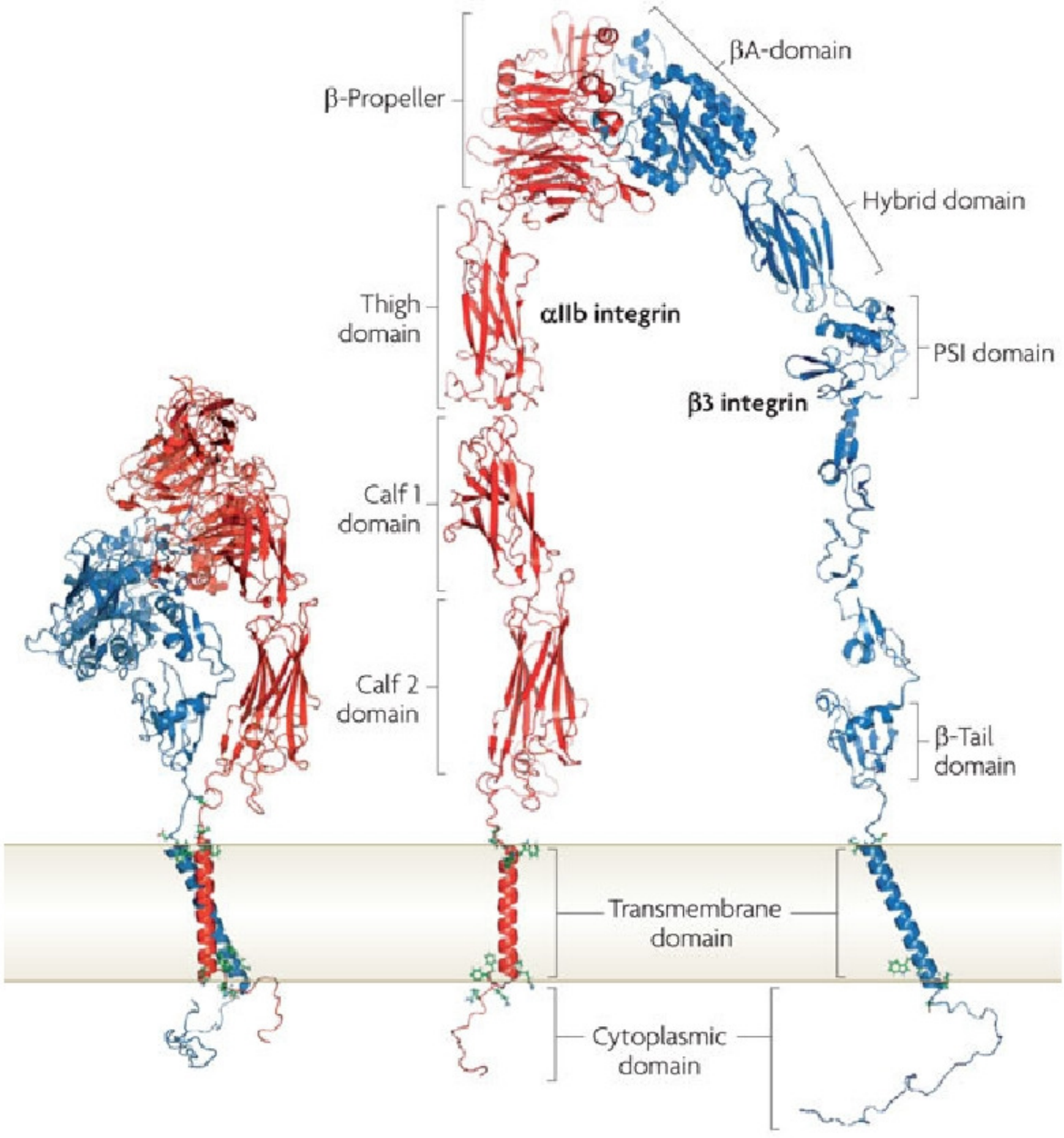

βA-domain
β-Propeller
Hybrid domain
Thigh domain
αIIb integrin
PSI domain
β3 integrin
Calf 1 domain
Calf 2 domain
β-Tail domain
Transmembrane domain
Cytoplasmic domain

Figure 1.4: Integrin structure. Integrins are heterodimeric adhesive receptors consisting of an α- and a β-subunit. In mammals, there are 24 canonical integrins formed from combinations of 18 α-subunits and 8 β-subunits. The 'bent conformation' seen in crystal structures (see the figure; left) can be unfolded to facilitate visualization of the domains (see the figure; right). In most integrins the amino-terminal domain in the α- and β-integrin subunits (the β-propeller and the βA domain, respectively), assemble by non-covalent interactions to form a 'head' and provide a ligand binding site. In 8 α-integrin subunits (α1, α2, α10, α11, αL, αM, αX and αD), the αA domain, which is homologous to the βA domain of the β-integrin subunit, is inserted into the β-propeller domain and is the main ligand-binding site in these integrins. In integrins that lack an A domain, such as $\alpha_{IIb}\beta_3$ integrin, which is depicted here, the βA domain forms the main ligand-binding site. Note that the plexin, semaphorin and integrin (PSI) domain is at the N terminus of the β-integrin subunit, but is joined by disulphide bonds to more carboxy-terminal residues. The remaining C-terminal extracellular domains of each subunit comprise two long 'legs'. The low affinity state of the integrin for its ligands is maintained by non-covalent interactions between the α- and β-integrin transmembrane and cytoplasmic domains. Figure and legend are reproduced from reference (Shattil et al., 2010).

1.2.4.2 Integrin signalling in platelet function

The cytoplasmic tails of integrin α and β chains bind various proteins that are involved in both "inside-out" and "outside-in" signalling. As described in section 1.2.3.2, "inside-out" signalling consists of the biochemical events that culminate in integrin activation. Although the mechanisms are still incompletely defined, recent advances have revealed that talin is a major mediator of integrin activation (Tadokoro et al., 2003). Talin comprises a C-terminal rod domain concealing an N-terminal head domain with high affinity for integrin β-chain cytoplasmic tails (Calderwood et al., 1999). Activated PLCβ and PLCγ2 raise platelet calcium and DAG levels (section 1.2.3.1). Subsequently, elevated intracellular calcium levels activate calpain, a protease which cleaves talin and liberates its head from the rod (Yan et al., 2001). Simultaneously, calcium and DAG activate a guanine nucleotide exchange factor (CalDAG-GEFI) that activates a Ras-family GTPase called Rap1b which also requires PI 3-K (Dupuy et al., 2001).

Rap1b then binds to an effector adaptor protein called Rap1–GTP-interacting adaptor molecule (RIAM) causing its migration to the membrane where it binds through PH-domains. RIAM also binds talin, recruiting it to the membrane, proximal to integrin cytoplasmic tails (Lafuente et al., 2004, Han et al., 2006). It is believed that integrins are constrained in inactive low-affinity conformations through salt-bridges holding α and β-chain cytoplasmic regions together (Hughes et al., 1996). Talin-binding disturbs these bonds, separates the chains and causes integrins to assume high affinity, active conformations. These events have been demonstrated for β_1, β_2 and β_3 integrins (Shattil et al., 2010). Other proteins have also

been shown to activate integrins through cytoplasmic tail binding including kindlins and β_3-endonexin (Shattil et al., 1995, Shattil et al., 2010). The principles of integrin "inside-out" signalling are outlined in Figure 1.5.

Extracellular ligand binding can itself activate integrins to cause "outside-in" signalling. This depends on integrin oligomerisation, also known as *clustering* which brings together various signalling proteins bound to cytoplasmic tails. Integrin clustering also increases the number and strength of interaction sites between platelets and ligands a process called *avidity modulation* (Shattil et al., 1998). Following α- and β-chain separation by "inside-out" signalling, ligand-binding causes α-chains to cluster with other α-chains and β-chains with other β-chains (homotypic oligomerisation). This has been demonstrated *in vitro* with Chinese Hamster Ovary (CHO) cells transfected with α_{IIb} and β_3 and shown to stimulate phosphorylation of the tyrosine kinase Src which is constitutively bound to β_3 (Obergfell et al., 2002, Buensuceso et al., 2003).

In resting integrins, associated Src is maintained in an inactive conformation by phosphorylation on an inhibitory site (Tyrosine^{529}) by c-Src kinase (Csk) which is also constitutively bound to β_3 (Obergfell et al., 2002). Ligand binding causes Src Tyrosine^{529} dephosphorylation by protein tyrosine phosphatase-1B (PTP-1B), Csk dissociation, Src autophosphorylation on an activatory site (Tyrosine^{418}) leading to its activation (Obergfell et al., 2002). Src then phosphorylates Tyrosine^{747} and Tyrosine^{759} residues on β_3 which serve as docking sites for the recruitment of Syk.

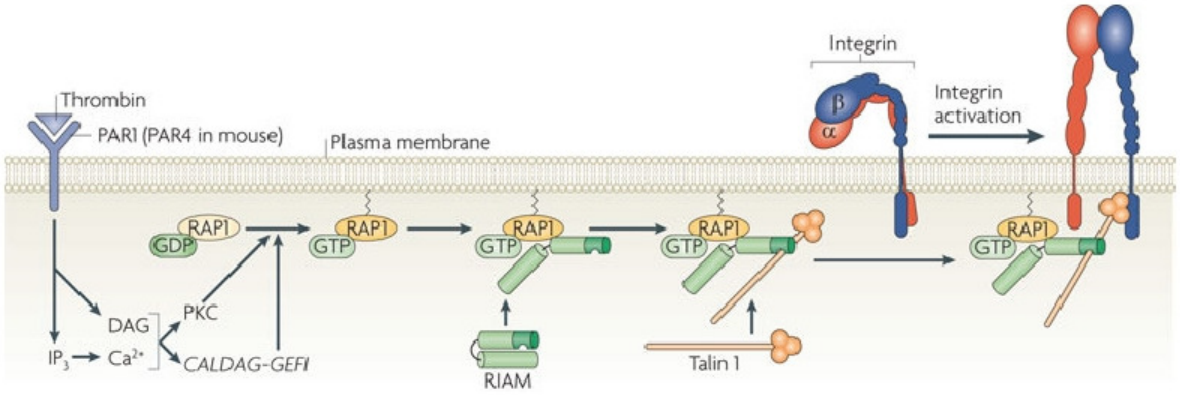


Figure 1.5: Integrin "inside-out signalling". The schematic represents the minimal elements of one pathway of $\alpha_{IIb}\beta_3$ integrin activation by thrombin receptors, which were identified through the synthetic reconstruction of pathway components in Chinese hamster ovary cells and studies of gene-targeted platelets. Thrombin cleavage or ligand occupancy of the thrombin receptor proteinase-activated receptor 1 (PAR1; also known as F2R) in human platelets, or PAR4 receptors in mouse platelets, stimulates phospholipid hydrolysis, which results in the generation of inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates an increase in cytosolic free Ca^{2+}, activating Ca^{2+}- and DAG-regulated (CALDAG-GEFI; also known as RASGRP1), which in turn converts its encoded protein, RAP1, from a GDP-bound to an active GTP-bound form. Ca^{2+} and DAG also activate certain protein kinase C (PKC) isoforms, including PKCα, which among other actions may facilitate the activation of CALDAG-GEFI. Activation of RAP1 leads to recruitment of its effector, RAP1–GTP-interacting adaptor molecule (RIAM; also known as APBB1IP), and its binding partner, talin 1, to the plasma membrane. This enables talin binding to the β_3 integrin tail and talin-induced activation of $\alpha_{IIb}\beta_3$ integrin. Kindlin 3 plays a crucial role in this process, but because its mechanistic role is uncertain, it is not depicted here. Figure and legend are reproduced from reference (Shattil et al., 2010).

Syk becomes activated by autophosphorylation and phosphorylates various proteins including the adaptor proteins SLP-76 and NcK, and the guanine exchange factor Vav1 (Figure 1.6) (Watson et al., 2005). SLP-76 is required for PLCγ2 activation which raises cellular calcium and activates PKC. Vav1 activates the Ras-family GTPases Rac and cdc42 which are involved in actin polymerisation, while Nck recruits Pak1, the effector protein for Rac. PI 3-K is also activated downstream of $\alpha_{IIb}\beta_3$ ligation and together with Src, activates focal adhesion kinases FAK which also mediates reorganisation of the actin cytoskeleton (Shattil and Newman, 2004).

1.2.5 Regulation of platelet activity

The luminal endothelial lining of blood vessels plays a major role in negatively regulating platelet activation which restricts the initiation of thrombus formation to vascular injury sites. Endothelial cells release nitric oxide (NO), in response to shear forces imposed by flowing blood (Furchgott and Zawadzki, 1980, Palmer et al., 1988). NO is a potent vasodilator that relaxes vascular smooth muscle and diffuses across platelet membranes to activate intracellular soluble guanylate cyclase (sGC) to produce cyclic guanosine monophosphate (cGMP). cGMP activates a dependent serine/threonine kinase; protein kinase G (PKG) which in turn mediates various inhibitory phosphorylation events (Radomski et al., 1987a, Radomski et al., 1987b, Feil et al., 2003).

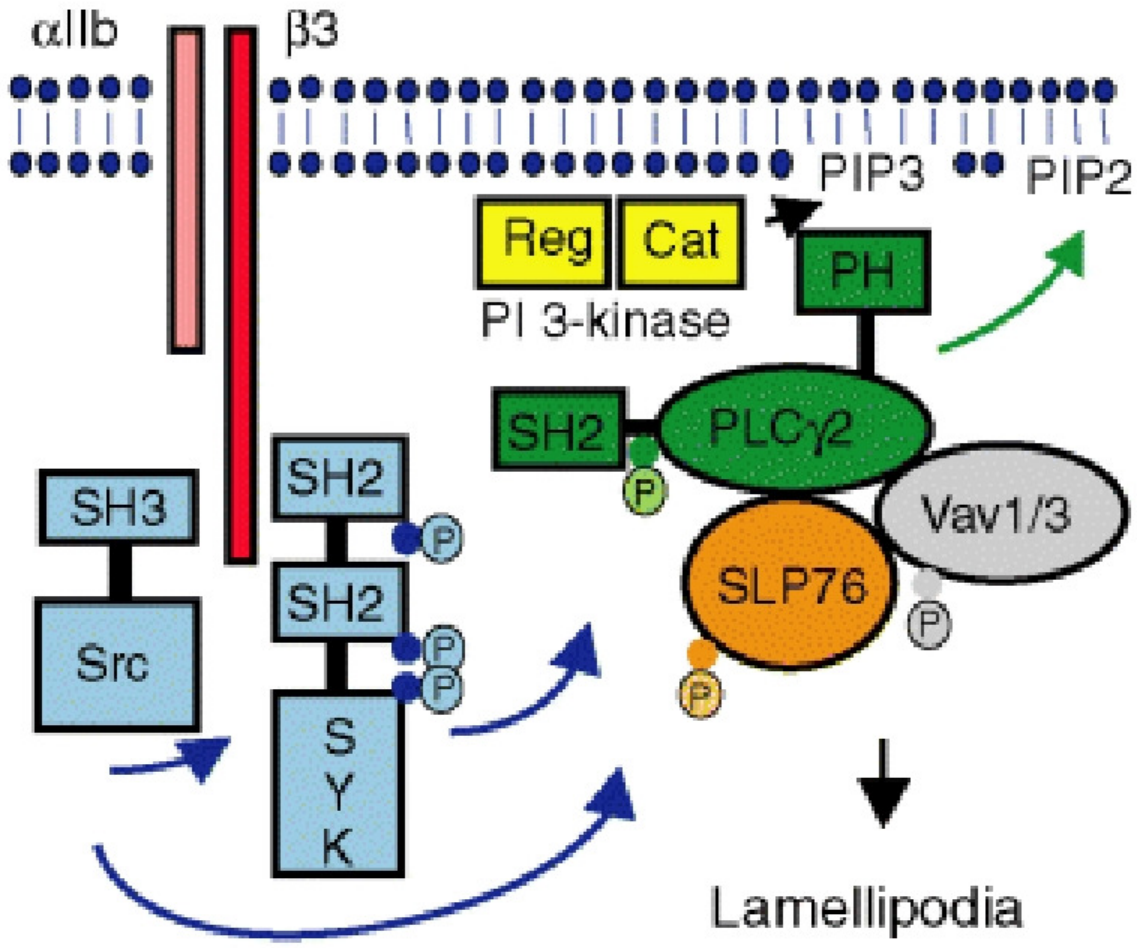


Figure 1.6: Integrin "outside-in signalling". Outside-in signalling through ligand engagement or clustering of integrin $\alpha_{IIb}\beta_3$ generates intracellular signalling cascades. Src-dependent activation of Syk leads to activation of PLCγ2 through a pathway that is likely to be dependent on SLP-76 and Vav. Figure and legend are reproduced in modified form from reference (Watson et al., 2005).

Notable among these are inhibition of Rap1b (Reep and Lapetina, 1996), TXA_2 receptors (Wang et al., 1998) and IP_3 receptors on the DTS (Schlossmann et al., 2000). Moreover, PKG activates sarcoendoplasmic reticulum calcium ATPase (SERCA) which pumps cytoplasmic calcium back into the DTS, returning its concentration to basal levels (Trepakova et al., 1999). Endothelial cells also release prostacyclin (PGI_2) which binds $G\alpha_s$-linked IP receptors on platelets. $G\alpha_s$ activates adenylate cyclase, which converts ATP to cAMP (Moncada et al., 1976, Gryglewski et al., 1976, Tateson et al., 1977, Dutta-Roy and Sinha, 1987). cAMP activates protein kinase A (PKA), a serine/threonine kinase that inhibits platelet TXA_2 synthesis and like PKG, also inhibits IP_3 receptors and Rap1 while activating SERCA (Schwarz et al., 2001).

CD39 and CD73 are ecto-5'-nucleotideases present on the endothelium which break down ADP to AMP and AMP to adenosine respectively, thus reducing platelet recruitment by diminishing ambient ADP levels (Marcus et al., 1997, Kawashima et al., 2000, Koszalka et al., 2004). Endothelial thrombomodulin binds plasma thrombin and alters its ligand specificity, diminishing its ability to activate platelets (Esmon et al., 1983). Furthermore, several phosphatases present within platelets reverse activatory phosphorylation events such as those occurring downstream of tyrosine kinases linked to GPVI and integrins (Cicmil et al., 2002, Newman and Newman, 2003).

1.2.6 Platelets in atherosclerosis

Atherosclerosis is a condition of chronic vascular inflammation implicated in many cardiovascular diseases which together are the greatest cause of mortality in the developed world (Ross, 1999). Fatty streaks gradually deposit on vascular lumens with age in a manner influenced by blood lipid levels. Despite its continuity, the endothelium is dynamic with small crevices continuously opening and closing between adjacent endothelial cells, through which cholesterol-rich low density lipoproteins (LDL) can enter the subendothelium (Gimbrone, 1999). Oxidative stress causes LDL oxidation by reactive oxygen species (ROS) such as hydroxyl ions (OH^-) and superoxide (O_2^-) to generate oxidised LDL (oxyLDL) which stimulates immunogenic responses by vascular dendritic and endothelial cells (Ross, 1999, Young and McEneny, 2001). This triggers monocyte recruitment to these regions where they differentiate into macrophages. Macrophages phagocytose oxyLDL, but due to their limited ability to process it, they transform into "foam cells". Various inflammatory mediators are released and concentrate at atherosclerotic sites, stimulating further monocyte recruitment.

In addition to their classical role in thrombosis, it has been suggested that platelets may also play a role in atherogenesis (Gawaz et al., 2005). OxyLDL and other inflammatory mediators cause endothelial dysfunction which is thought to result in a phenotype where the endothelium produces less PGI_2 and NO, releases vWF from Weibel-Palade bodies and expresses various cell adhesion molecules. This leads to platelet accrual on its surface, where they further aggravate endothelial dysfunction (Ware and Heistad, 1993, Andre et al., 2000). Platelets release proinflammatory mediators including CD40 ligand, PDGF, interleulkin-1β,

PF_4 and transforming growth factor β (TGF-β) which enhance monocyte recruitment (Figure 1.7) (Henn et al., 1998, Gawaz et al., 2005). Platelets also bind monocytes directly through GP1b/IX/V-mediated interactions with monocyte P-selectin glycoprotein ligand-1 (PSGL-1) and integrin $\alpha_M\beta_2$ (Mac-1) (Simon et al., 2000). Inflammatory mediators also stimulate vascular smooth muscle to proliferate, produce collagen and encapsulate subendothelial lipid material forming atherosclerotic plaques (Ross, 1999). These plaques are subsequently broken down and weakened by matrix metalloproteinases released by platelets and macrophages (Fernandez-Patron et al., 1999). Shear forces imposed by blood or other physical pressure may eventually rupture the plaques and expose platelets to prothrombotic collagen fibres leading to vascular occlusion (Gawaz et al., 2005). Atherosclerosis is implicated in various morbidities including peripheral vascular disease (PVD), MI and strokes. Targeting platelet function has proven an effective clinical strategy in controlling mortality due to these pathologies as evidenced by successful interventions with anti-platelet drugs such as aspirin, clopidogrel and $\alpha_{IIb}\beta_3$ receptor antagonists (Lincoff et al., 2000, Bhatt et al., 2006).

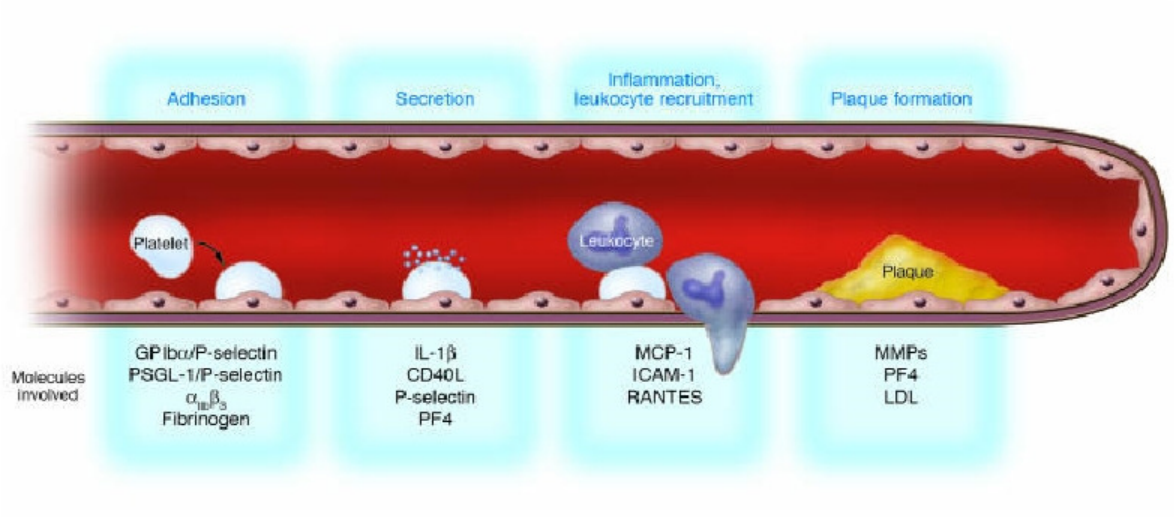


Figure 1.7: **Hypothetical model of atherogenesis triggered by platelets.** Activated platelets roll along the endothelial monolayer via GPIbα/P-selectin or PSGL-1/P-selectin. Thereafter, platelets firmly adhere to vascular endothelium via β_3 integrins, release pro-inflammatory compounds (IL-1β, CD40L), and induce a proatherogenic phenotype of endothelial cells (chemotaxis, MCP-1; adhesion, ICAM-1). Subsequently, adherent platelets recruit circulating leukocytes, bind them, and inflame them by receptor interactions and paracrine pathways, thereby initiating leukocyte transmigration and foam cell formation. Thus, platelets provide the inflammatory basis for plaque formation before physically occluding the vessel by thrombosis upon plaque rupture. Figure and legend are reproduced in modified form from reference (Gawaz et al., 2005).

1.3 The coagulation cascade

The haemostatic system is a physiological response to injury that is initiated to limit blood loss and preserve bodily homeostasis. The rapid activation of platelets and their roles at sites of vascular injury constitutes *primary haemostasis*. However, the initial haemostatic plug is fragile, easily dislodged by flowing blood and requires stabilisation. Hence, a parallel system is simultaneously initiated to reinforce and stabilise the developing platelet plug. This *secondary haemostasis* involves a series of complex proteolytic reactions that culminate in the formation of a stable fibrin clot. The majority of enzymes involved are vitamin K-dependent serine proteases and their glycoprotein cofactors synthesised in the liver.

The sequential nature of protein activation during secondary haemostasis has led to the term *coagulation cascade*. This has become well established since its inception in 1964 (Davie and Ratnoff, 1964, Macfarlane, 1964). Thrombin is the main effector protein of the cascade and two pathways lead to its generation. The first is the *intrinsic (contact activation) pathway*, so named as all the mechanisms required to activate it are endogenously present in the blood and it can be initiated *in vitro* following blood contact with negatively charged surfaces. The second is the *extrinsic (tissue factor) pathway* which is triggered by blood exposure to subendothelial tissue factor (TF). However, a constitutive presence of potentially thrombogenic plasma TF under normal conditions has been reported (Giesen et al., 1999). Moreover, secretion of TF by monocytes and platelets has also been described (Osterud, 1998, Rauch et al., 2000, Siddiqui et al., 2002). Both pathways converge at a final *common*

pathway through which thrombin is activated from its zymogen precursor, prothrombin (Figure 1.8). Critical to the progression of the coagulation cascade is the spatial localisation of mediators involved in some of the key reactions. This occurs at the surface of activated platelets and is facilitated by their exposure of negatively charged phosphatidylserine which provides the essential reactive surface (Heemskerk et al., 2002). The major components of the coagulation cascade are called *coagulation factors* and they are named by their designated roman numerals prefixed with the letter "F" for "factor". Their activated forms are suffixed by the letter "a".

In the intrinsic pathway, high molecular weight kininogen (HMWK) and prekallikrein serve as cofactors for the activation of FXII to generate FXIIa which then activates FXI. FXIa in turn activates FIX. In the presence of calcium and its cofactor FVIIIa, FIXa transforms FX to FXa at the platelet surface in what is called the *tenase complex*. The route to the tenase complex via the extrinsic pathway is triggered by TF binding to FVIIa. The TF-FVIIa complex then directly causes FX activation at the platelet surface. In a calcium dependent reaction, FXa associates with its cofactor FVa in cleaving prothrombin (FII) to form thrombin (FXIIa). This is referred to as the *prothrombinase complex* which executes the common pathway of thrombin activation. Thrombin cleaves fibrinopeptides A and B from Aα and Bβ fibrinogen chains respectively causing them to assemble in an insoluble fibrin polymer that reinforces platelet thrombi. Thrombin also activates FXIII which stabilises fibrin through covalent transglutaminase cross-linking reactions.

The description above is a simplified outline of the inherent events. Considerable crosstalk occurs between the pathways since FVIIa can activate FIX and thrombin can activate FXI, FV, FVIII and platelets (Pieters et al., 1989, Gailani and Broze, 1991). The cascade is also tightly controlled with the principal regulator being antithrombin III, a plasma protein which inhibits the serine proteases of the cascade (Olson et al., 1993). Its affinity is raised by endothelial surface heparin sulphates and therapeutically by heparin. Thrombomodulin on the endothelium also alters thrombin substrate specificity resulting in its activation of protein C which inhibits FVa and FVIIIa (Esmon et al., 1982, Esmon et al., 1993).

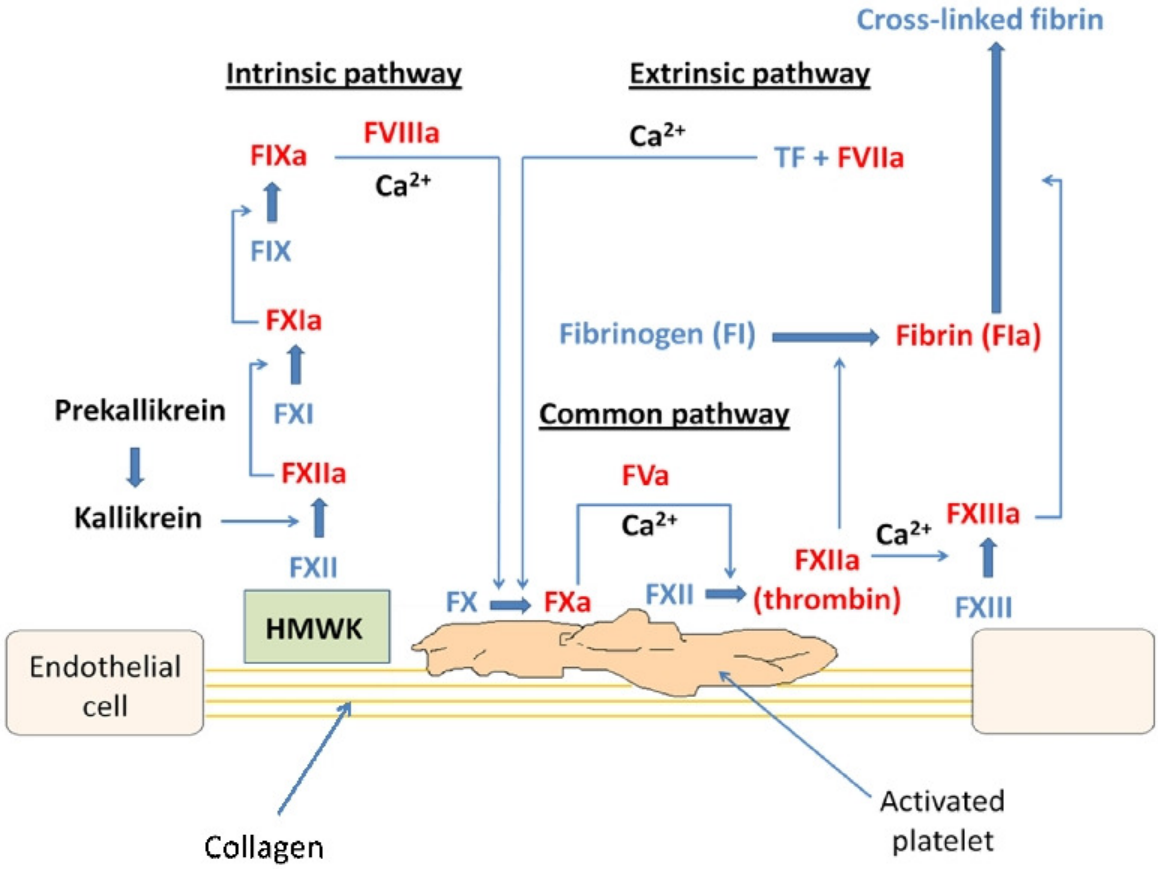


Figure 1.8: The blood coagulation cascade. Haemostasis is the body's physiological response to vascular injury and involves formation of a primary occlusive platelet plug (primary haemostasis) which is stabilised by a fibrin gel formed through a series of events mediated by serine proteases (secondary haemostasis) which constitute the coagulation cascade. Thrombin is the main effector enzyme of the cascade as it cleaves fibrinogen to form fibrin, activates the transglutaminase FXIII to cross-link and stabilise fibrin and is also the major agonist stimulating platelet aggregation. Thrombin (FIIa) is activated from its precursor zymogen (prothrombin, FII) in the common pathway of the coagulation cascade which requires phosphatidylserine exposure by activated platelets. As shown in the figure, two pathways converge on the common pathway. These are the intrinsic (contact activation) pathway and extrinsic (tissue factor) pathway. The intrinsic pathway is stimulated by contact of high molecular weight kininogen (HMWK) with negatively charged surfaces such as negative species in the ECM while the intrinsic pathway is initiated by tissue factor (TF) associating with FVIIa. Further descriptive details are provided in the text.

1.4 Factor XIII

FXIII belongs to the transglutaminase family of enzymes which join peptide chains through ε(γ-glutamyl)lysine cross-links (Folk, 1983). FXIII-deficiency was first identified as a cause of clinical bleeding in a young boy in 1960 (Duckert et al., 1960), and in 1963, FXIII received formal recognition as a blood coagulation factor.

1.4.1 FXIII structure

FXIII circulates in plasma comprised of two globular, catalytic A-subunits (FXIII-A) encapsulated by two strand-like, carrier B-subunits (FXIII-B) giving it a tetrameric structure (A_2B_2). FXIII-A is a 731 amino acid protein of 83kDa encoded by a gene on chromosome 6 (Board et al., 1988). It is divided into 5 well-characterised domains: an N-terminal activation peptide, beta sandwich, catalytic core, barrel 1 and barrel 2 at the C-terminus (Yee et al., 1994). FXIII-A is expressed by haematopoietic cells and has been found in megakaryocytes, platelets, monocytes, macrophages, and their precursor stem cells (Muszbek et al., 1996, Adany and Bardos, 2003). Intracellular FXIII-A is found in the cytoplasm in dimeric form (A_2) and is thought to be released into plasma following cell destruction. FXIII-A has 9 cysteine residues, with at least one disulphide bond (Takahashi et al., 1986). Its free thiols may mediate a recently described protein disulphide isomerase (PDI) activity (Lahav et al., 2009).

FXIII-B is a glycoprotein produced by hepatic cells (Muszbek et al., 1996). It is 641 amino acids long, 80kDa and its gene lies on chromosome 1 (Webb et al., 1989). Unlike FXIII-A, FXIII-B has a signal sequence and is secreted into blood where it complexes with FXIII-A. In plasma, the

presence of FXIII-B in 50% excess over FXIII-A ensures that all FXIII-A is in complex form with FXIII-B, protecting it from premature activation (Yorifuji et al., 1988). FXIII-B consists of ten 60 amino acid sequence repeats called "sushi domains" (Ichinose et al., 1986). These are known to facilitate protein-protein contacts and may mediate FXIII-B dependent FXIII-binding to C-terminal ends of fibrinogen γ'-chains in plasma (Siebenlist et al., 1996). The variant γ'-chains have a 20 amino acid C-terminal extension and are found in 15% of plasma fibrinogen (Wolfenstein-Todel and Mosesson, 1980). FXIII-B is also believed to stabilise FXIII-A in plasma maintaining its half-life at about 10 days. In the absence of FXIII-B, FXIII-A is rapidly depleted from plasma with its half-life reduced to around 3 days (Saito et al., 1990).

1.4.2 Activation of FXIII

Similar to other coagulation factors, FXIII circulates in plasma as an inactive zymogen; only becoming activated at sites of vascular injury. Activation of FXIII is triggered by thrombin-mediated cleavage of a 4kDa N-terminal activation peptide from FXIII-A at arginine^{37}-glycine^{38}. B-subunits then dissociate in a calcium-dependent manner, exposing a free thiol at cysteine^{314} in A-subunit catalytic sites, allowing them to engage substrate molecules (Lorand, 1986) (Figure 1.9). Fibrinogen greatly enhances B-subunit dissociation, thus facilitating FXIII activation (Naski et al., 1991).

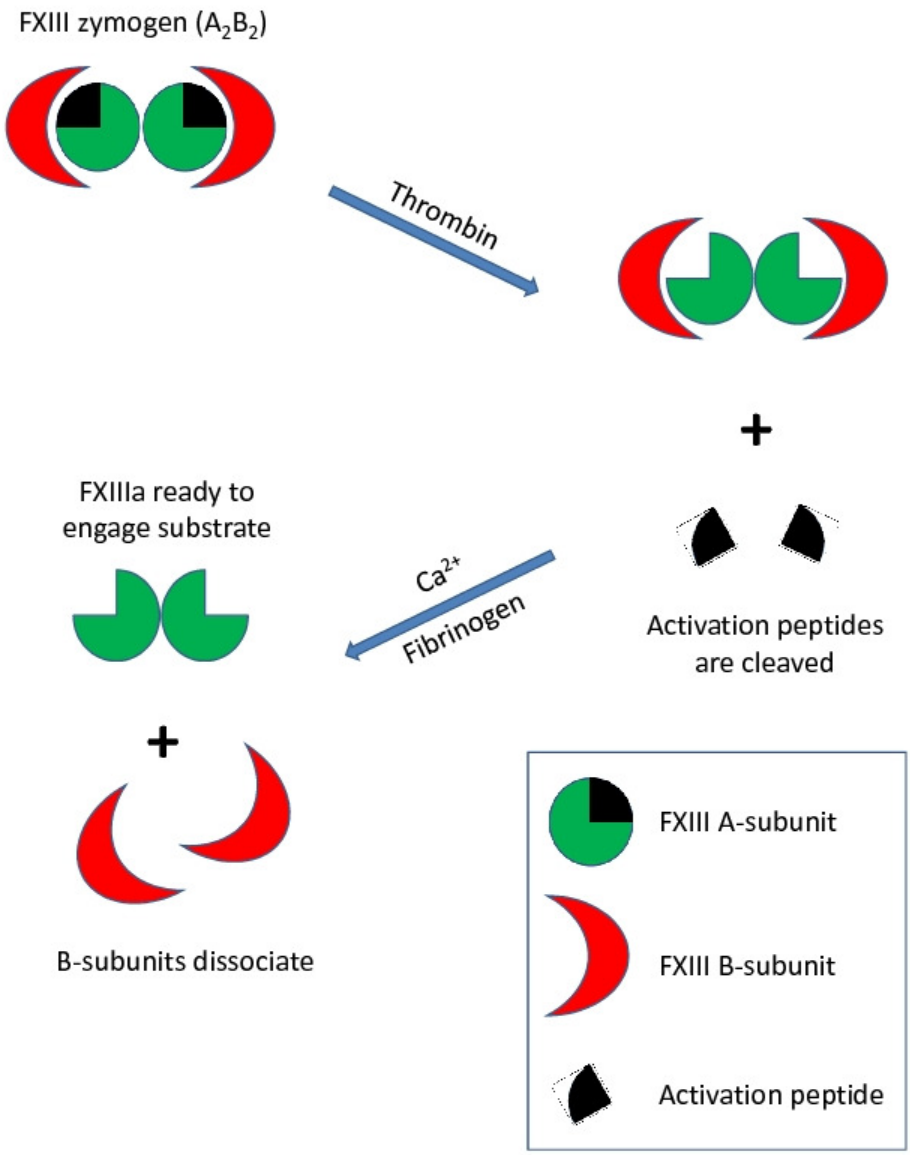


Figure 1.9: Structure and activation of coagulation factor XIII. Factor XIII (FXIII) circulates in plasma as a tetramer comprising of 2 A-subunits and 2 B-subunits. The carrier B-subunits bound to the catalytic A-subunits prevent premature enzyme activation maintaining the half-life of FXIII at about 10 days. Activation of FXIII follows thrombin-mediated cleavage of activation peptides from A-subunits. B-subunits then dissociate from the complex in a calcium-dependent step which is greatly enhanced by fibrinogen. Activated FXIII (FXIIIa) then engages its substrates to catalyse ε(γ-glutamyl)lysine cross-links.

The exposed cysteine[314] on activated FXIII (FXIIIa) associates with a substrate γ-glutamine through an intermediate thioester bond. This rate-limiting step releases ammonia which is reduced in the plasma under physiological conditions. Although there is substantial heterogeneity in protein sequences surrounding substrate γ-glutamines selected by transglutaminases (Griffin et al., 2002), it is surmised that the charge and structure around them as determined by primary structure may be involved (Muszbek et al., 1996). γ-glutamines are transferred to ε-lysine residues linking the two in isopeptide bonds. This covalently joins two peptide chains while leaving FXIIIa free to engage more substrate molecules (Figure 1.10).

Cellular FXIII-A is thought to become activated independently of proteolytic cleavage following intracellular calcium elevation that accompanies cell activation (Polgar et al., 1990). Since FXIII-B is not expressed by haematopoietic cells and is therefore absent, calcium is not required for B-subunit dissociation in this case. Rather, it is hypothesised that intracellular calcium mediates a slow progressive conformational change in FXIII-A through which it exposes its reactive cysteine.

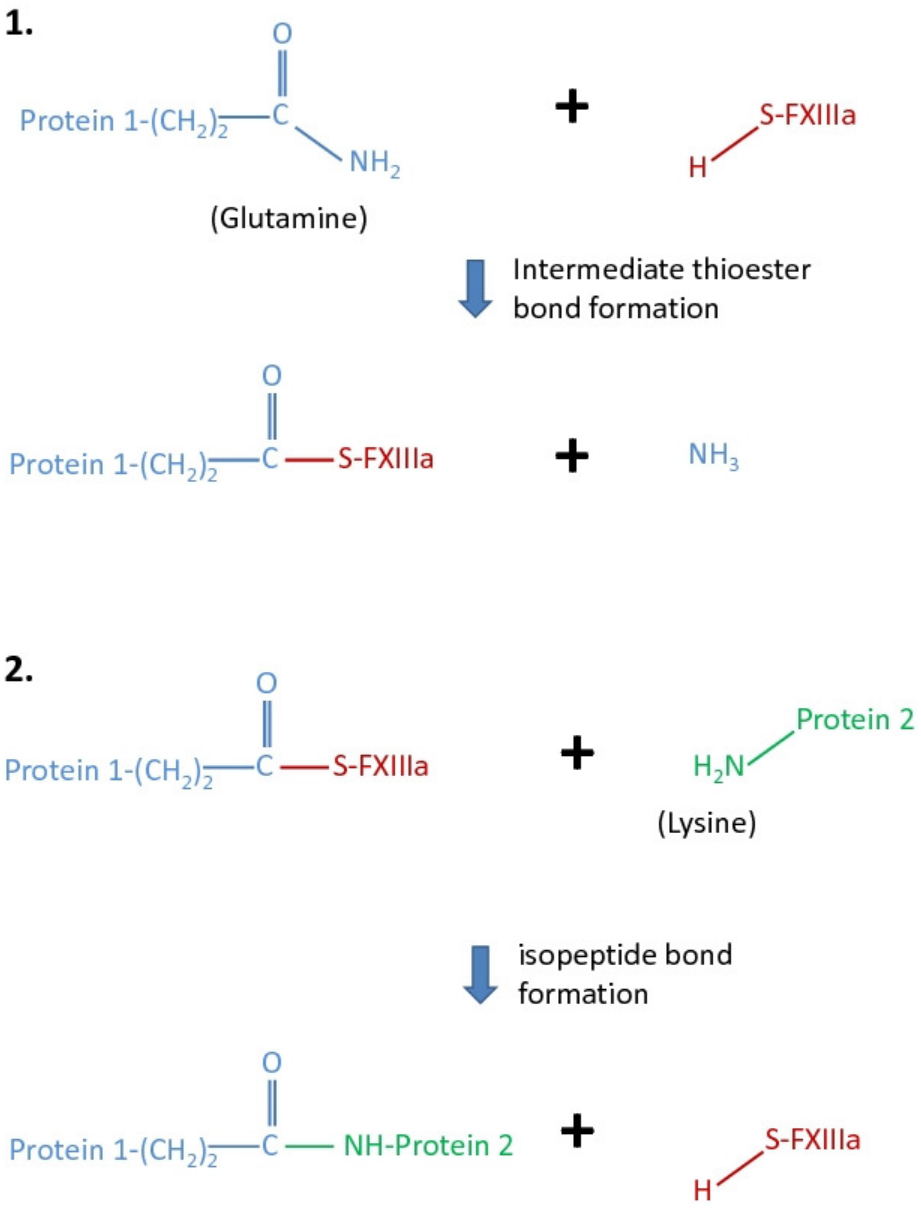


Figure 1.10: Transglutaminase reaction of FXIIIa. **(1)** Activated factor XIII (FXIIIa) exposing its catalytic thiol at cysteine[314] initially forms a thioester bond with a protein-bound glutamine residue such as that on protein 1 in the figure. This rate-limiting reaction releases ammonia. **(2)** Through the thioester intermediate, FXIIIa transfers the glutamine residue to a primary amine from a protein-bound lysine residue, resulting in an isopeptide bond. FXIIIa then emerges free to engage more substrates.

1.4.3 Functions of FXIII

FXIIIa plays a critical role in haemostasis through stabilising fibrin clots formed by the coagulation cascade, where its activation constitutes the final step (Figure 1.8). FXIIIa cross-links both fibrin and fibrinogen, however, the reaction with fibrinogen is slower (Kanaide and Shainoff, 1975). Fibrin(ogen) α and γ-chains are joined through the transglutaminase reaction illustrated in Figure 1.10 (Mosesson, 1997). The γ-chains are rapidly cross-linked into dimers whereas α-chains are more slowly assembled into multimers. In addition, FXIIIa also incorporates other proteins into fibrin(ogen) clots. Importantly, α_2-antiplasmin, a major antagonist to the fibrinolytic enzyme plasmin, is cross-linked to fibrin(ogen) α-chains thus protecting clots from premature degradation (Sakata and Aoki, 1982). This latter activity of FXIIIa has been described as its most important haemostatic role (Muszbek et al., 1999). It is believed that FXIIIa imparts early clot stability through γ-chain cross-linking while long-term resistance to shear forces of flowing blood and fibrinolytic degradation are attained through α-chain cross-linking and α_2-antiplasmin incorporation (Muszbek et al., 1999). Other anti-fibrinolytic agents that are incorporated into clots by FXIIIa include thrombin activatable fibrinolysis inhibitor (TAFI) and plasminogen activator inhibitor type 2 (PAI-2) (Muszbek et al., 2008).

FXIIIa is also involved in wound healing and about 15% of FXIII-deficient patients are limited in this respect (Lorand et al., 1980). FXIII-A deficient mice displayed markedly limited wound-healing which was alleviated by FXIII supplementation (Inbal et al., 2005). Moreover, human supplementation with FXIII concentrates hastened the healing of wounds in patients who

had undergone surgery for removal of head and neck tumours (Brockmeier et al., 1998). Mediation of wound-healing is believed to occur through both direct cell binding and reorganisation of the ECM. Fibroblasts critically facilitate wound healing by producing ECM proteins that form a scaffold for reparative scars. Surface-bound FXIII increases fibroblast proliferation (Bruhn and Pohl, 1981). In addition, fibroblasts adhere to immobilised FXIIIa in an integrin-dependent manner that stimulates activatory signal transduction and shape changes in these cells (Ueki et al., 1996). Through cross-linking ECM proteins such as collagen, fibronectin, vWF and thrombospondin as well as incorporating plasma proteins such as fibrinogen into the ECM, FXIIIa alters its structure, thereby providing a more favourable surface for fibroblast migration (Corbett et al., 1997, Grinnell et al., 1980, Muszbek et al., 1996).

FXIIIa was also shown to bind endothelial cells through $\alpha_v\beta_3$, stimulating their proliferation and migration in a manner dependent on its transglutaminase activity (Dardik et al., 2003). Proliferation and migration of endothelial cells are instrumental in angiogenesis which also aids wound healing. However, other investigators reported that FXIIIa-endothelial interactions inhibit angiogenesis independently of transglutaminase activity (Dallabrida et al., 2000). These contradictory findings reveal the complexity of FXIII-cellular interactions, but may be due to differences in experimental methods between studies.

FXIII-deficient women cannot carry a pregnancy to term (Ichinose et al., 2005), since FXIII is required for the formation of the Nitabuch's layer and the cytotrophoblastic shell which enable placental attachment to the uterus (Asahina et al., 2000). Uterine macrophages are

believed to be the source of FXIII in this organ, yet plasma supplementation with FXIII concentrate alleviates spontaneous abortions by FXIII-deficient women. Thus, the mechanisms of FXIII delivery to the uterus are still poorly understood.

1.4.4 Regulation of FXIIIa activity

Endogenous mechanisms that lead to the demise of FXIIIa activity are poorly defined. In rabbits, FXIIIa-mediated fibrinogen α-chain cross-linking was shown to continue for over 6 hours (Finlayson and Aronson, 1974), and α_2-antiplasmin cross-linking to clots had a half-life of 20min (Robinson et al., 2000). Since thrombin substrate-specificity is altered by thrombomodulin on the endothelial surface, it has been suggested that this may reduce thrombin-mediated FXIIIa cleavage (Muszbek et al., 1999, Philippou et al., 2003).

Catani et al., (1998) demonstrated *in vitro* inhibition of FXIIIa by NO. They suggested that NO reacts with the reactive cysteine of FXIIIa through a nitrosylation reaction which inhibits its interaction with substrates (Catani et al., 1998). Although NO is a physiological antagonist that is constantly secreted into blood, FXIIIa inhibitory effects were only observed at supraphysiological levels of NO in that study. Inhibitory FXIII-A cleavage by proteases released by neutrophils including elastase and cathepsin-G have also been described as possible regulatory mechanisms (Bagoly et al., 2007). Since association with FXIII-B constrains FXIII-A activity, FXIII-B may potentially inhibit plasma FXIIIa. However it is yet to be tested whether FXIII-B can reassemble with FXIIIa *in vivo*. Thus, although a number of potential biochemical mechanisms capable of regulating the activity of FXIIIa have emerged, evidence

of whether they are able to operate in the complex and dynamic *in vivo* environment is still insufficient.

A common Valine^{34}Leucine polymorphism in the FXIII-A gene present in 25% of Caucasians is activated faster by thrombin cleavage (Muszbek, 2000). Leucine^{34} was shown to interact more avidly with hydrophobic sequences in the thrombin substrate-binding site than Valine^{34} thus facilitating its cleavage at Arginine^{37}-Glycine^{38} (Trumbo and Maurer, 2000, Trumbo and Maurer, 2003). While Leucine^{34} has a faster rate of activation, at full activation, both enzyme forms have equal specific transglutaminase activity. Paradoxically, the variant Leucine^{34} has been shown to be protective against MI (Suzuki et al., 1996, Kohler et al., 1998), with this being dependent on fibrinogen concentration (Muszbek et al., 2008). There is no protective effect at low fibrinogen concentration, however, at high concentration, Leucine^{34} cross-links fibrin into a loose and open mesh that is more prone to fibrinolysis (Lim et al., 2003).

1.4.5 FXIII deficiency

The average human plasma FXIII (A_2B_2) concentration is 21.6µg/mL (0.07µM) (Yorifuji et al., 1988). However, normal plasma enzyme concentration ranges widely from 66% to 134% (Katona et al., 2000), while enzyme activity ranges from 53% to 221% (Anwar et al., 1999). Surprisingly, less than 5% of the average plasma enzyme concentration level is sufficient to allay bleeding (Walls and Losowsky, 1968). Congenital FXIII deficiency is a heritable disorder which follows autosomal recessive genetics (Anwar and Miloszewski, 1999). It is a rare condition with a morbidity of 1 in 3-5 million and is caused by severe reduction or absence of the functional enzyme in platelets and plasma of affected individuals (Board et al., 1993).

Patients with mutations in genes encoding FXIII-A (chromosome 6p24-25) and FXIII-B (chromosome 1q31-32.1) resulting in failure to express either protein have been described, however, the majority of cases involve abnormalities in FXIII-A (Anwar and Miloszewski, 1999). Since FXIII is activated in the terminal stage of the coagulation cascade, after formation of the fibrin clot, its deficiency is not detectable using standard tests of haemostatic function. Thus, prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time are normal in FXIII deficient patients. The disease is instead detected by testing for rapid clot solubility in 5M urea or 1% monochloroacetic acid which is indicative of FXIII deficiency. This can then be followed by more sophisticated quantitative analyses.

The first clinical case of FXIII deficiency was documented by Duckert et al, (1960) and over the years, the symptoms of the disease have become well characterised. The most distinctive symptom (appearing in 80% of cases) is umbilical bleeding occurring a few days after birth (Anwar and Miloszewski, 1999). Although FXIII levels are typically lower in neonates than in adults, under normal conditions they are sufficient to prevent bleeding. Other symptoms include post-operative bleeding, intramuscular and joint haemorrhage and superficial bruising. Poor wound healing has been described, but is not a universal symptom, reported in only 14% of patients (Anwar and Miloszewski, 1999). Intracranial haemorrhage is a more widespread symptom (30% of patients) and the major cause of mortality in affected patients (Anwar et al., 2002). All symptoms are managed by supplementation with FXIII concentrate (Anwar and Miloszewski, 1999, Hsieh and Nugent, 2008). Currently, plasma-derived FXIII

concentrate is indicated for this purpose, but recombinant FXIII concentrate has been developed and is undergoing clinical trials (Lovejoy et al., 2006, Hsieh and Nugent, 2008).

FXIII deficiency can also be an acquired trait, whereby individuals born with a capacity to produce normal amounts of FXIII experience a pathological decline in their plasma levels. This may be caused by depletion of FXIII and other coagulation factors through disseminated intravascular coagulation (DIC). Alternatively, abnormal synthesis of "inhibitor" auto-antibodies which attack the host's own FXIII-A has been described (Board et al., 1993). Production of inhibitor antibodies may be spontaneous, drug-induced or be caused by autoimmune disease. Additionally, liver disease may result in less production of carrier FXIII-B (Muszbek et al., 1996), causing a reduction in the plasma half-life of FXIII-A (Saito et al., 1990).

1.4.6 The roles of platelet intracellular FXIII

Approximately half of the FXIII-A found in the body resides as dimers (A_2) within the platelet cytoplasm, where it is present at a concentration between 100-150 times greater than in plasma (Buluk, 1955, Muszbek et al., 1996). A small amount of tetrameric FXIII (A_2B_2) is found in platelet α granules (Marx et al., 1993), which is thought to be passively absorbed from plasma as platelets permeate the vasculature. Granular FXIII (A_2B_2) may be released during platelet activation and thus increase local FXIIIa concentrations at sites of thrombosis.

The mechanisms involved in the release of platelet cytoplasmic FXIII (cFXIII) to plasma are not well understood, although it is assumed that this occurs following platelet destruction

(Lopaciuk et al., 1976). There is widespread consensus that platelets are the major sources of plasma FXIII-A (Muszbek et al., 1999). However, a radical view that monocytes, not platelets perform this role has recently been proposed (Cordell et al., 2010). The investigators of that study reported normal plasma FXIII-A levels in thrombocytopenic mice. However, the mice used in the study possessed residual blood platelets which given their high cytoplasmic FXIII-A content, may still have been able to replenish plasma levels of the enzyme. Moreover, the direct secretion of FXIII-A by monocytes/macrophages was not demonstrated.

Despite its intracellular abundance, the roles of platelet cFXIII are poorly established. Several studies have reported that activated platelet cytoplasmic FXIII (cFXIIIa) can cross-link various intracellular substrates (Cohen et al., 1981, Serrano and Devine, 2002, Adany and Bardos, 2003). Following platelet activation, elevated cellular calcium is thought to elicit cFXIII activation through slow, progressive conformational change (Polgar et al., 1990, Muszbek et al., 1993). Elements of the cytoskeleton including actin, myosin and vinculin were demonstrated to be cFXIIIa substrates, fuelling speculation of its involvement in cytoskeleton stability and clot retraction (Cohen et al., 1981). Zhu et al., (1994) surmised that the small heat-shock protein HSP27 associates with cFXIIIa and transports it to the cytoskeleton where it performs cross-linking reactions.

Surprisingly, Kulkarni and Jackson (2004) showed that cFXIIIa negatively regulated $\alpha_{IIb}\beta_3$ and mediated the development of a procoagulant platelet phenotype under conditions of prolonged elevated intracellular calcium levels. These results were contradicted by a more recent study where FXIII-A deficient platelets showed reduced $\alpha_{IIb}\beta_3$-mediated fibrinogen

binding (Jayo et al., 2009). Furthermore, spreading responses were also diminished, supporting hypothetical involvement of cFXIIIa in cytoskeletal organisation (Jayo et al., 2009). Notwithstanding these findings, there is still limited evidence supporting contributions of cFXIIIa to *in vivo* platelet function.

1.4.7 Platelet interactions with plasma FXIII

In elegant studies involving swapping normal and FXIII-A deficient platelets with FXIII-A deficient and normal plasma respectively, platelets were revealed to greatly influence FXIII activity (Francis and Marder, 1987, Reed et al., 1992). While the presence of normal platelets in FXIII-A deficient plasma resulted in fibrin cross-linking, it was much less than that induced by platelet-depleted normal plasma. This suggested that although platelet FXIII-A may be released, it plays a modest role in cross-linking fibrin. However, the mere presence of platelets in normal plasma, whether FXIII-A deficient or not, greatly accelerated FXIIIa-mediated fibrin cross-linking compared to platelet-depleted plasma (Hevessy et al., 1996). This can be explained by the fact that platelets provide a reactive surface which facilitates thrombin generation. Thrombin then mediates FXIII activation by proteolytic cleavage.

Adhesive interactions between platelets and exogenous FXIII were first described in 1984 by Greenberg and Shuman who reported that platelets activated uniquely by thrombin attained a FXIII-binding site on their surfaces (Greenberg and Shuman, 1984). They further suggested that the interactions were mediated by FXIII-A not FXIII-B, required FXIII activation, but not its activity and occurred independently of $\alpha_{IIb}\beta_3$. The latter was deduced from normal FXIII-binding by platelets from people with Glanzmann's thrombasthenia (GT) which lack

functional $\alpha_{IIb}\beta_3$ on their surfaces. This observation was however contradicted ten years later by a study demonstrating major dependency of FXIII-platelet adhesive interactions on $\alpha_{IIb}\beta_3$ integrins using multiple approaches including the use of platelets from people with GT (Cox and Devine, 1994). In the same study, the investigators also showed that platelets could be stimulated to bind FXIIIa by a broader range of agonists and not just thrombin alone (Cox and Devine, 1994). Nevertheless, the physiological roles of platelet FXIII-binding remained poorly defined.

Recently, platelet-dependent clot retraction was found to be severely compromised in FXIII-A deficient mice (Kasahara et al., 2010). This is consistent with an earlier study using human platelets where clot retraction was found to be critically dependent on platelet $\alpha_{IIb}\beta_3$, fibrin(ogen) and FXIIIa transglutaminase activity (Cohen et al., 1982). However, it is unclear whether platelet-FXIII interactions are directly involved or rather that FXIIIa stabilises already retracted clots by cross-linking fibrin. In any case, FXIII-A deficient mice were shown to suffer extended bleeding times due to unstable thrombi (Lauer et al., 2002).

Although FXIII binds platelet $\alpha_{IIb}\beta_3$, its involvement in platelet aggregation is unclear. While an early study described normal *in vitro* aggregation to ADP, ristocetin and collagen (Ozsoylu and Hicsonmez, 1976), a more recent investigation using platelets from 3 FXIII-A deficient patients showed abnormal responses to collagen, ADP and epinephrine (Anwar et al., 2002). In addition, by incorporating fibronectin into fibrin at developing thrombi, FXIIIa enhanced human platelet aggregation under flow (Cho and Mosher, 2006). FXIIIa was also revealed to be involved in the formation of highly thrombogenic "COAT" platelets which bear released α-

granule proteins cross-linked to serotonin on their membrane surfaces (Dale et al., 2002). However, later work using murine platelets could not establish a role for FXIIIa in this process (Jobe et al., 2005), possibly due to another transglutaminase fulfilling this role in mice. Thus, despite wide recognition of platelet-FXIII interactions and their potential implications to platelet function, understanding of their physiological roles remains limited.

1.4.8 Platelet-FXIII interactions in cardiovascular disease

Through its transglutaminase-mediated clot stabilisation, FXIII imparts fibrinolytic resistance to preformed clots. This has rejuvenated interest in the study of its involvement in the pathogenesis of cardiovascular disease since it may affect patient responses to fibrinolytic therapy (Muszbek et al., 1999). However, little is known about potential pathological consequences of platelet-FXIII associations.

Platelets from patients with PVD were observed to circulate with higher levels of bound FXIII than matched healthy controls (Devine et al., 1993). This observation was important for two reasons. Firstly, it demonstrated that platelet-FXIII interactions occur *in vivo.* Secondly, it suggested that these interactions may have pathological implications. Indeed, plasma FXIII was found to mediate abnormal adhesion of stimulated platelets to dysfunctional endothelium by binding to $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ respectively (Dardik et al., 2002a). This suggests potential roles for FXIII in accentuating pathological platelet-endothelial interactions that hallmark atherosclerotic progression. Certainly, when considered in unison with the involvement of platelets in the progression of atherosclerosis (section 1.2.6), the fact that women with elevated plasma FXIII antigen and activity levels are predisposed to PVD

(Shemirani et al., 2008) and MI (Bereczky et al., 2007) demands further investigation of platelet-FXIII interactions to clarify their roles in cardiovascular pathologies.

1.5 Aims of the study

It is established that blood platelets play a key role in the formation of haemostatic plugs at sites of vascular injury. This occurs through their interaction with a number of adhesive ligands generally in the form of proteins found in the extracellular matrix. More recently, it has been demonstrated that proteins involved in the coagulation cascade such as FXI and protein C may also contribute to platelet entrapment upon vascular injury (White et al., 2008, White-Adams et al., 2009). These findings suggest a potentially new level of interaction between primary and secondary haemostatic systems.

FXIII plays a key role in the haemostatic process through the cross-linking of fibrin monomers. Moreover, it is also known to adhere to platelet surfaces, although the importance of this to platelet function has remained elusive. The present project tested the hypothesis that immobilised FXIII stimulates functional responses in adherent platelets which influence their roles in haemostasis. In order to achieve this greater understanding of platelet-FXIII interactions, the experiments performed were designed to fulfil a number of aims:

- To determine the ability of immobilised FXIII to support platelet adhesion and spreading

- To examine the ability of FXIII to support adhesion and thrombus formation under conditions of flow

- To clarify the roles of FXIII enzymatic activity in this process

- To identify which receptor(s) are responsible for the interactions between FXIII and platelets.

- To dissect the signal transduction pathways activated by FXIII in platelets

- To determine if FXIII can potentiate the thrombogenic potential of established platelet-adhesive ligands

CHAPTER 2 - MATERIALS AND METHODS

2.1 Antibodies

Normal mouse IgG, anti-phosphotyrosine, anti-LAT, anti-SLP76, anti-$\alpha_v\beta_3$ (LM609) and anti-β-tubulin monoclonal antibodies were purchased from Millipore (Watford, UK). Rabbit monoclonal anti-PLCγ2 antibody and mouse monoclonal anti-Syk were purchased from Santa Cruz (Heidelberg, Germany). Fluorescein Isothiocyanate (FITC)-conjugated mouse monoclonal anti-fibrinogen and anti-P-selectin antibodies were from BD Biosciences (Oxford, UK). Mouse monoclonal and polyclonal anti-FXIII-A, FITC-conjugated goat anti-mouse antibody, and rabbit polyclonal anti-fibrinogen antibody were from Abcam Plc (Cambridge, UK).

2.2 Chemicals and reagents

Phe-Pro-Arg-Chloromethylketone (PPACK) was purchased from Cambridge Bioscience Ltd (Cambridge, UK). H-Arg-Gly-Asp-Ser-OH (RGDS), 1,2-bis-(o-aminophenoxy)ethane-tetra-acetic acid tetra-(acetoxymethyl)ester (BAPTA-AM), phorbol 12-myristate 13-acetate (PMA), (Ro-31-8200, bisindoylmaleimide I (BIM I) and bisindoylmaleimide V (BIM V) were all purchased from Calbiochem, UK. 4-Amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]-pyrimidine (PP1) was purchased from BIOMOL International (Exeter, UK). U73122 was purchased from Tocris Bioscience (Bristol, UK). Prestained molecular weight protein ladder was from New

England Biolabs (Ipswich, UK). Collagen horm reagent was purchased from Nycomed (Zurich, Switzerland). Purified vWF (LFB; Lyon, France) and purified, human plasma-derived FXIII (Fibrogammin P; CLS Behring, UK) were generously donated by the Haematology Unit at Bradford Royal Infirmary (Bradford, UK). Tirofiban (Aggrastat; Iroko Pharmaceuticals, USA) was a kind gift from the Haematology Unit at Castle Hill Hospital (Hull, UK). Recombinant fibrinogen purified from culture media of Chinese Hamster Ovary cells stably transfected with the three chains of fibrinogen as previously described (Ajjan et al., 2008), was provided by Dr. Ramzi Ajjan (University of Leeds, UK). Dr. Ajjan also provided human recombinant FXIII-A (rFXIII-A) expressed in BL21-Gold DE3 *E. coli* (Stratagene, UK). Pooled, heat-inactivated human serum (HS) was from Sheffield University Hospital, (Sheffield, UK). Human α-thrombin, ethyleneglycoltetraacetic acid (EGTA), bovine serum albumin (BSA), bacitracin and all other reagents were purchased from Sigma (Poole, UK).

2.3 Methodology used in the activation of FXIII

2.3.1 *In vitro* activation of FXIII

Plasma-purified FXIII in Fibrogammin P concentrate was reconstituted to a final concentration of 500U/mL (1.1mg/mL), while rFXIII-A was provided at a stock concentration of 300μg/mL. Both FXIII preparations were activated by incubation with human α-thrombin (10U/mL) in the presence of $CaCl_2$ (10mM) for 1h at 37°C. The reaction was terminated by a 10 minute incubation with PPACK (20μM) at 37°C to inactivate thrombin (Dardik et al., 2002b). Complete inhibition of thrombin under these conditions was confirmed by assessing its diminished ability to stimulate platelet shape change and aggregation.

2.3.2 Measurement of FXIII transglutaminase activity

FXIII activity was monitored by measuring the cross-linking of biotinylated amines to fibrinogen through adapting a previously described method (Song et al., 1994). Wells of a 96-well microplate were coated with fibrinogen (40µg/mL) in Tris-buffered saline (TBS; 40mM Tris, 140mM NaCl, pH 8.3) for 45min at room temperature on an orbital shaker. The wells were then emptied and uncoated surfaces blocked with BSA/TBS (1%) for 1h at room temperature. After blocking, wells were washed three times with TBS. The following were then added at the indicated final reactive concentrations: FXIII or rFXIII-A (both 22µg/mL), 5-(biotinamido)pentylamine (1mM), dithiothreitol (0.5mM), $CaCl_2$ (100mM) and thrombin (10U/mL). Thrombin cleaved FXIII/rFXIII-A which became activated in the presence of calcium ions. Dithiothreitol maintained exposure of catalytic thiols in the activated enzymes, allowing them to cross-link 5-(biotinamido)pentylamine to fibrinogen immobilised on the wells which was allowed to proceed at room temperature. The reaction was stopped after 10min by addition of Ethylenediaminetetraacetic acid (EDTA, 1mM) to chelate calcium ions. Wells were then washed 3 times with TBS-Tween (0.01%). Streptavidine-alkaline phosphatase (1µg/mL in 0.1% BSA/TBS) was subsequently added to conjugate specifically with fibrinogen-bound 5-(biotinamido)pentylamine. This reaction was allowed to proceed for 1h at room temperature on a shaker. The wells were subsequently washed 3 times with TBS-Tween (0.01%) and incubated with *p*-nitrophenylphosphate (1mg/mL in 1M diethanolamine, pH 9.8, containing 0.5mM $MgCl_2$) for 30min at 37°C. The latter was converted to yellow *p*-nitrophenol following hydrolysis of a phosphate group by alkaline phosphatase. Colour development was stopped by addition of NaOH (4M) and optical density measured at 405nm using a microplate reader.

In some instances, FXIIIa and rFXIIIa activated as described in section 2.3.1, were inhibited by incubation with the alkylating agent iodoacetamide (3mM) for 30 minutes at 22°C (Dardik et al., 2003). Alternatively, both proteins were heat-denatured by incubation in a water-bath at 60°C for 1 hour. Inhibition of transglutaminase activity under each of these conditions was assessed by carrying out the activity assay described above.

2.4 Methodology used for the preparation of platelets

Platelets were prepared from whole blood obtained from healthy, adult volunteers. Exclusion criteria were smoking, chronic illness, and taking medication within 14 days prior to bleeding. In all cases, informed consent was obtained in accordance with the Declaration of Helsinki. Ethical permission for the use of human blood in this project was granted by the ethics committe at the Postgraduate Medical institute (Hull York Medical School, Hull, UK). Blood was acquired by ante-cubital venepuncture using a 21-gauge butterfly needle. The first 3mL were discarded to exclude activated platelets. Subsequently, blood was collected into 20mL syringes and mixed with acid citrate dextrose (ACD; 29.9mM sodium citrate, 113.8mM glucose, 72.6mM NaCl and 2.9mM citric acid, pH 6.5) 5:1 (v/v) as anticoagulant (Riba et al., 2008).

2.4.1 Isolation of platelets from whole blood

Blood was centrifuged at 200*g* at 20°C for 20 min. This caused its separation into 3 layers; an upper layer of platelet-rich plasma (PRP) suspended over a narrow band of leukocytes (buffy coat) which overlaid a layer of red blood cells packed at the bottom of the centrifugation

tube. Washed platelets were prepared using the prostaglandin E_1 (PGE_1) method adapted from Vargas et al., (1982). This method relies on transient platelet inhibitory effects mediated by PKA which is activated following PGE_1-induced cAMP elevation. Avoiding the buffy coat, PRP was carefully isolated with a Pasteur pipette then centrifuged at 800*g* at 20°C for 12 min in the presence of PGE_1 (50ng/mL), to pellet suspended platelets. Depending on pellet size, platelets were resuspended in 1-2mL of modified Tyrode's buffer (150mM NaCl, 5mM HEPES, 0.55mM NaH_2PO_4, 7mM $NaHCO_3$, 2.7mM KCl, 0.5mM $MgCl_2$ and 5.6mM glucose, pH 7.4) and left to recover from PGE_1 treatment for 1 hour prior to use.

2.4.2 Isolation of red blood cells from blood

Red blood cells (RBC) were pelleted from whole blood during the isolation of PRP described above (section 2.4.1). After removing PRP supernatant, the leukocyte-rich buffy coat was also carefully removed and discarded. RBCs were resuspended in 30mL of wash buffer (10mM EDTA, 50mM glucose, 90mM NaCl, 36mM citric acid, 50mM KCL, pH 6.5) and washed twice by sequential buffer replacement and centrifugation at 800*g*. A final wash was carried out with 30mL of modified Tyrode's buffer. When required, RBCs were reconstituted with autologous washed platelets in modified Tyrode's buffer to final concentrations of 50% (v/v) and 2×10^8 platelets/mL respectively.

2.4.3 Quantification of platelet numbers

Platelet quantification was performed manually using a haemocytometer. This device is a specialised microscope slide on which perpendicular lines have been engraved to form two grids in a central chamber burrowed 0.1mm beneath the surface of one side (Figure 2.1). When a cover-slip is placed over the chamber, the resulting confined space holds a defined volume of cell suspension 0.1mm above the grids. Cells are allowed time to settle and then counted under a phase contrast microscope.

A sample of washed platelet suspension was diluted 1:100 in ammonium oxalate solution (1%, w/v). This concentration of ammonium oxalate lyses any red blood cells present while platelets remain intact (Brecher and Cronkite, 1950). After thorough mixing followed by incubation for 10 minutes, the diluted platelet suspension was loaded onto the haemocytometer to cover each grid and left for a further 10 minutes to allow platelets to settle over the grids prior to counting.

Each of the two grids engraved on a haemocytometer is divided into 9 large squares measuring 1mm × 1mm (1 mm^2). The central 1mm^2 square is further divided into 25 smaller squares, each with an area of 0.04mm^2. After loading the haemocytometer with diluted platelet suspension, 10 of the 0.04mm^2 squares were chosen for platelet counting from each grid as shown by an "X" in Figure 2.1. Platelet numbers obtained from both grids were averaged to give the mean number of platelets in ten 0.04mm^2 squares (mean number of platelets/0.4mm^2).

Since the height of the platelet suspension above the grids was 0.1mm and volume = area × height, it follows that this number of platelets was suspended in; $0.4mm^2 \times 0.1mm = 0.04mm^3$.

Considering that $1mm^3 = 10^{-3}$ mL and by letting the mean number of counted platelets be x;

There were x platelets/$0.04mm^3$ = (x/0.04) platelets/mm^3 = (x/0.04) platelets/10^{-3} mL

= [(x/0.04) × 10^3] platelets/mL = 25000x platelets/mL in the diluted sample.

Thus, the concentration of platelets in the original suspension prior to a 1:100 dilution in ammonium oxalate

= (25000 × 100 × x) platelets/mL

= 25x × 10^5 platelets/mL

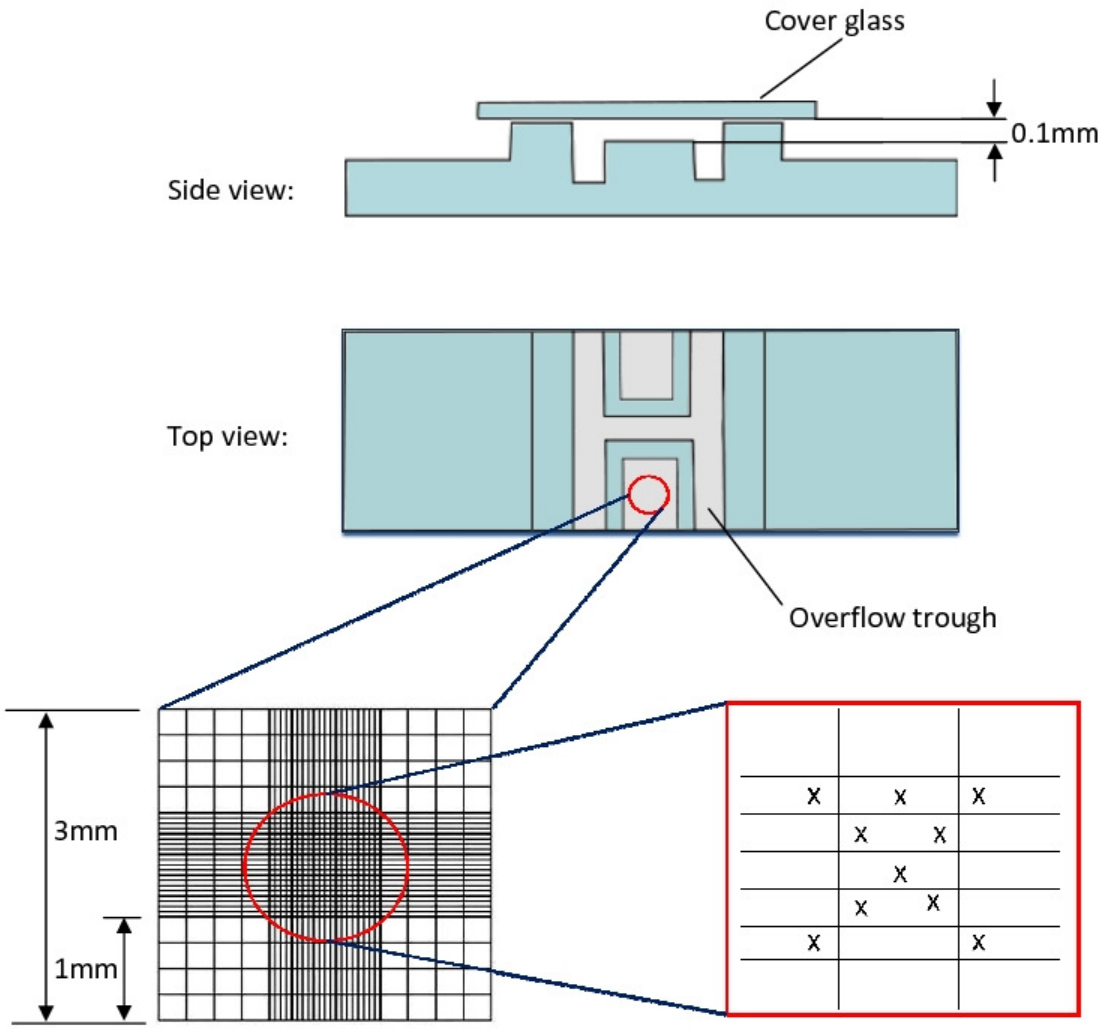


Figure 2.1: A haemocytometer. Platelets were counted from the squares marked with an "X" on each of the two grids

2.5 Methodology for the examination of platelet adhesion under static conditions

During the project, platelet adhesion was studied under various conditions. Initial experiments sought to establish and characterise the nature of platelet adhesion to FXIII under static conditions with a view to progress into more physiological, flow-based models. Adhesion experiments involved quantitative analysis of platelets bound to surface-immobilised adhesive proteins using either a microplate reader or fluorescence microscopy.

2.5.1 Microplate-based platelet adhesion assay

An adhesion assay adapted from Bellavite et al., (1994) was used to examine adhesive interactions between platelets and exogenous FXIII. In this assay, wells of a microplate are coated with platelet adhesive proteins and uncoated regions blocked with serum albumin. Platelets are allowed to adhere to the coated wells and non-adherent platelets are washed out. A buffered solution containing detergent for membrane lysis and *p*-nitrophenylphosphate is then added to the wells. Platelets contain the enzyme acid-phosphatase in their lysosomes which catalyses the hydrolysis of a phosphate group from *p*-nitrophenylphosphate. The reaction produces yellow *p*-nitrophenol which is optically dense and maximally absorbs light with a wavelength of 405nm. Colour development is stopped by adding alkali solution and optical density is then measured with a plate reader. The intensity of the colour obtained is proportional to the amount of enzyme and consequently, the total number of platelets present (Bellavite et al., 1994).

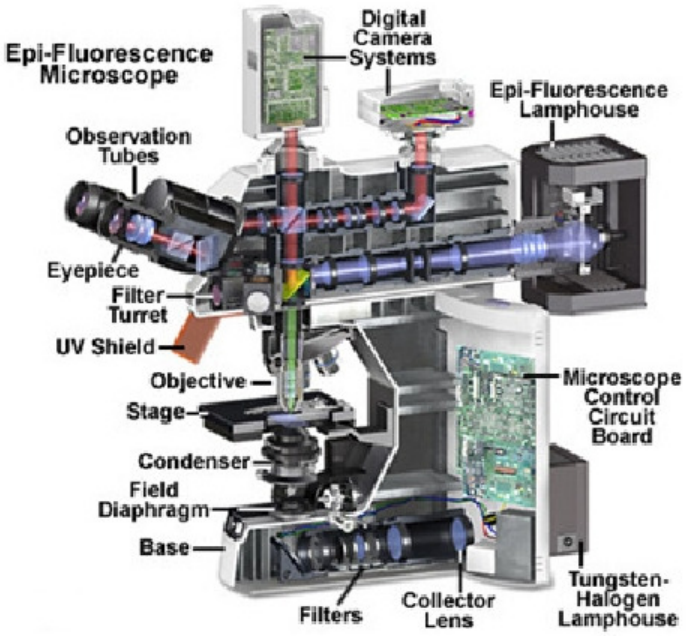
FXIII or FXIIIa (both 200µg/mL) or human serum (HS, 5%) were used to coat 96-well microplates overnight at 4°C. Residual coating protein was removed by two washes with phosphate buffered saline (PBS) and the wells subsequently blocked for 30min at room temperature with HS (5%). In some experiments, platelets were preincubated with the required inhibitors for 20min at 37 °C. Washed platelets (1×10^8 platelets/mL) were added to each well and left to adhere at 37 °C for 1 hour. Non-adherent platelets were emptied and the wells washed twice with PBS. Adherent platelets were incubated with reaction buffer (31mM citric acid, 5mM sodium citrate dehydrate, 5mM *p*-nitrophenylphosphate, 0.1% (v/v) Triton X-100, pH 5.4) for 1 hour at room temperature. The reaction was stopped by addition of NaOH (2M) to each well and optical density measured at 405nm.

2.5.2 Fluorescence microscopy

A fluorescence microscope takes advantage of the ability of some materials to discharge light to facilitate their experimental observation and analysis. Although some substances such as chlorophyll, crystals and vitamins can autofluoresce, most require staining with special fluorescent dyes called fluorochromes to enable visualisation. Fluorochromes are particularly useful when they selectively stain structures within a biological specimen, allowing specific analysis. For example, 3,3'-Dihexyloxacarbocyanine iodide ($DiOC_6$) and mepacrine stain platelet endoplasmic reticulum (Terasaki, 1989) and dense granules (Skaer et al., 1981) respectively. Where a specific fluorochrome for a given biological structure is unavailable, a generic one can be conjugated to an antibody generated against that structure, thereby producing a specific fluorescent stain.

Fluorochromes maximally absorb light of specific wavelength, resulting in their electrons gaining energy to migrate from ground energy states to excited states. However, they rapidly lose this energy by giving it off as light of longer wavelength, thereby returning to their ground energy states. This principle was fist postulated by Sir George Stokes in 1852 and has become known as "Stokes shift". A fluorescence microscope detects low energy light emitted by fluorochromes after separating it from the high energy light irradiated for their excitation by guiding it through wavelength-selective filters. Emitted light is guided towards the microscope eye-piece or a specialised digital camera, allowing visual observation and capture of images for further analysis (Figure 2.2A).

A)



B)

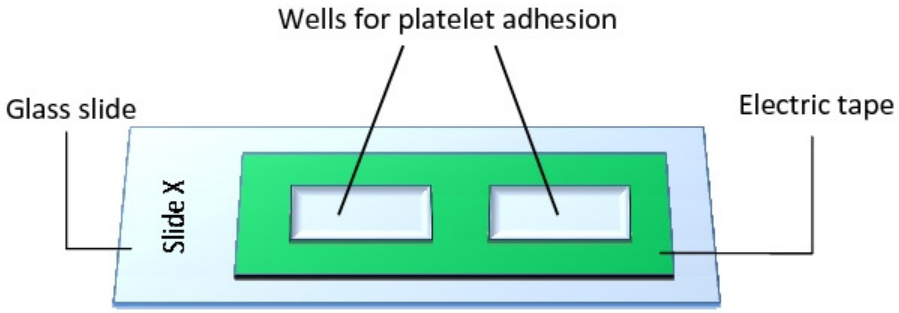


Figure 2.2: Equipment used to visualise fluorescently-stained platelets adherent on a protein-coated surface. **(A)** An illustration showing a fluorescence microscope, highlighting the separation of sample excitation and emission light through a system of wavelength-specific filters. Image from reference (Spring and Davidson, 2010). **(B)** Wells measuring 0.5cm × 1cm (0.5cm^2) were cut into electric tape adhered to glass microscope slides. The wells were coated with required proteins to study adhesion of fluorescently-labelled platelets using a fluorescence microscope.

2.5.2.1 Platelet adhesion to immobilised proteins under static conditions

Platelet adhesion under static conditions was also assessed using fluorescence microscopy. For this method, platelets were adhered to proteins coated on microscope slides. Adherent platelets were then stained with a fluorochrome and visualised under a microscope.

Specific areas of microscope slides (0.5cm×1cm) were coated overnight with HS (5%), plasma-purified FXIII (200µg/mL), FXIIIa (200µg/mL), recombinant FXIII (rFXIII, 200µg/mL), activated recombinant FXIII (rFXIIIa, 200µg/mL), or fibrinogen (200µg/mL). Residual protein was removed by washing slides in phosphate buffered saline (PBS). Uncoated glass surfaces were blocked by HS (5%) for 30min at room temperature. Washed platelets (5×10^7 platelets/mL) were then adhered for 1 hour at 37°C. Slides were processed as previously described (Roberts et al., 2008). Briefly, non-adherent platelets were removed by washing with PBS then stably adherent platelets were fixed with 4% paraformaldehyde in PBS for 30min. Adherent platelets were then permeabilised with 0.3% Triton X-100 for 7min and F-actin was stained with Tetramethyl Rhodamine Iso-Thiocyanate (TRITC)-conjugated phalloidin (10µg/mL) for 1 hour to enable visualisation under fluorescence. The platelets were subsequently overlaid with histomount under a cover-slip. Slides were observed with an Olympus IX71 fluorescence microscope equipped with an XM10 CCD camera (Olympus, Japan). Images were captured under a ×60 oil immersion objective lens and analysed using ImageJ software from the National Institutes of Health (NIH, USA). For each experiment, platelet numbers from random visual fields with a total area of $0.1mm^2$ were added and

results calculated as mean number of adherent platelets ± standard deviation (SD) per $0.1mm^2$.

In some experiments, platelets were preincubated with U73122 (5μM) to inhibit phospholipase C, BAPTA-AM (20μM) to chelate intracellular Ca^{2+}, apyrase (1U/mL) to scavenge ADP, indomethacin (10μM) to block thromboxane A_2 (TXA_2) synthesis, PP1 (20μM) to inhibit Src-family kinases, GSNO (10μM) to inhibit platelet activation, RGDS (500μM) or EGTA (1mM) to broadly inhibit platelet integrins, anti-$\alpha_v\beta_3$ (LM609, 20μg/mL) to block $\alpha_v\beta_3$, tirofiban (2μM) to specifically inhibit $\alpha_{IIb}\beta_3$ function or modified Tyrode's buffer as a control. Incubations were for 20 minutes at 37°C.

2.5.2.2 Analysis of granular secretion by adherent platelets

Platelet adhesion to various proteins is often followed by activatory responses such as shape change, spreading and granular secretion. Platelet α-granules contain proteins including P-selectin and fibrinogen which become more abundant on the platelet surface following activation-dependent degranulation. These proteins can be detected by probing adherent platelets with specific fluorochrome-conjugated antibodies which enable visualisation under a fluorescence microscope.

To investigate whether secretion occurred in FXIIIa-adherent platelets, platelets were adhered to FXIIIa-coated slides as described in section 2.5.2.1. However, lysis of platelet membranes with 0.3% Triton X-100 was omitted and adherent platelets were covered with 1% BSA/PBS for 30 minutes to block non-specific antibody binding. The presence of secreted

P-selectin and fibrinogen on adherent platelet surfaces was examined by probing for each protein using the respective specific FITC-conjugated antibodies (1:100 in 1% BSA/PBS) for 1h at room temperature. Unbound antibodies were washed off with PBS and the slides covered with histomount under a cover-slip. Images were then captured using a ×60 objective of a fluorescence microscope under oil immersion.

2.5.2.3 Adhesion of FXIII to collagen

Interactions between FXIII(a) and immobilised collagen were analysed using methodology adapted from a study investigating FXII binding to collagen (van der Meijden et al., 2009). Wells on glass slides were coated with collagen (100µg/mL) overnight at 4°C. After removal of unbound collagen with PBS, BSA (10mg/mL) was used to block uncoated surfaces for 30min at 22°C. The wells were then overlaid with FXIII (200µg/mL) or FXIIIa (200µg/mL) for 15 minutes at 37°C. After rinsing with PBS, collagen-bound FXIII or FXIIIa were probed with a primary mouse monoclonal antibody (1:100 in PBS containing 1% BSA) specific for FXIII-A for 1hour at 22°C. Unbound antibody was washed off with PBS. The wells were subsequently covered with secondary FITC-conjugated anti-mouse antibody (1:2000 in PBS containing 1% BSA). After thorough washes to remove excess antibody, cover-slips were placed over the slides and fluorescent images were captured under oil immersion using a ×100 objective lens.

2.6 Analysis of platelet adhesion under flow

While useful as first-line methodology to establish possible platelet interactions with various exogenous components, platelet adhesion assayed under static conditions is limited in its

simulation of physiological events. Platelets circulate in flowing blood. Therefore, interactions between platelet receptors and their ligands occur within a haemodynamic environment. The velocity of blood moving in a vessel is not uniform. It is lowest nearest to the vessel wall, due to frictional forces opposing the direction of flow and highest at the centre of the vessel lumen (Kroll et al., 1996). This gradation in speed of flow between alternate fluid layers creates a shearing effect. Considering that this shearing effect is dually dependent on blood velocity and distance from the wall, its quantification (the shear rate) is measured in centimetres moved per second per centimetre from the wall, which simplifies to inverse seconds (s^{-1}).

In the normal circulation, blood is under high pressure from the heart in the arterial circulation where it travels at its highest velocity with estimated shear rates ranging from 500 to 5,000s^{-1} (Tangelder et al., 1988). Even higher shear rates of up to 20,000s^{-1} have been reported in stenotic vessels (Siegel et al., 1994). In contrast, blood is under lower pressure in the venous system where it relies on skeletal muscle contraction and a system of valves to return it to the heart. Shear rates in this circulation range from 20 to 200s^{-1} (Kroll et al., 1996).

2.6.1 Platelet adhesion to FXIII under flow

The method of Cooke *et al*., (1993), modified as previously published from the laboratory (Roberts et al., 2009), was used to study platelet adhesion under flow. This involved real-time imaging of thrombus formation by fluorochrome-stained platelets using fluorescence microscopy (Figure 2.3).

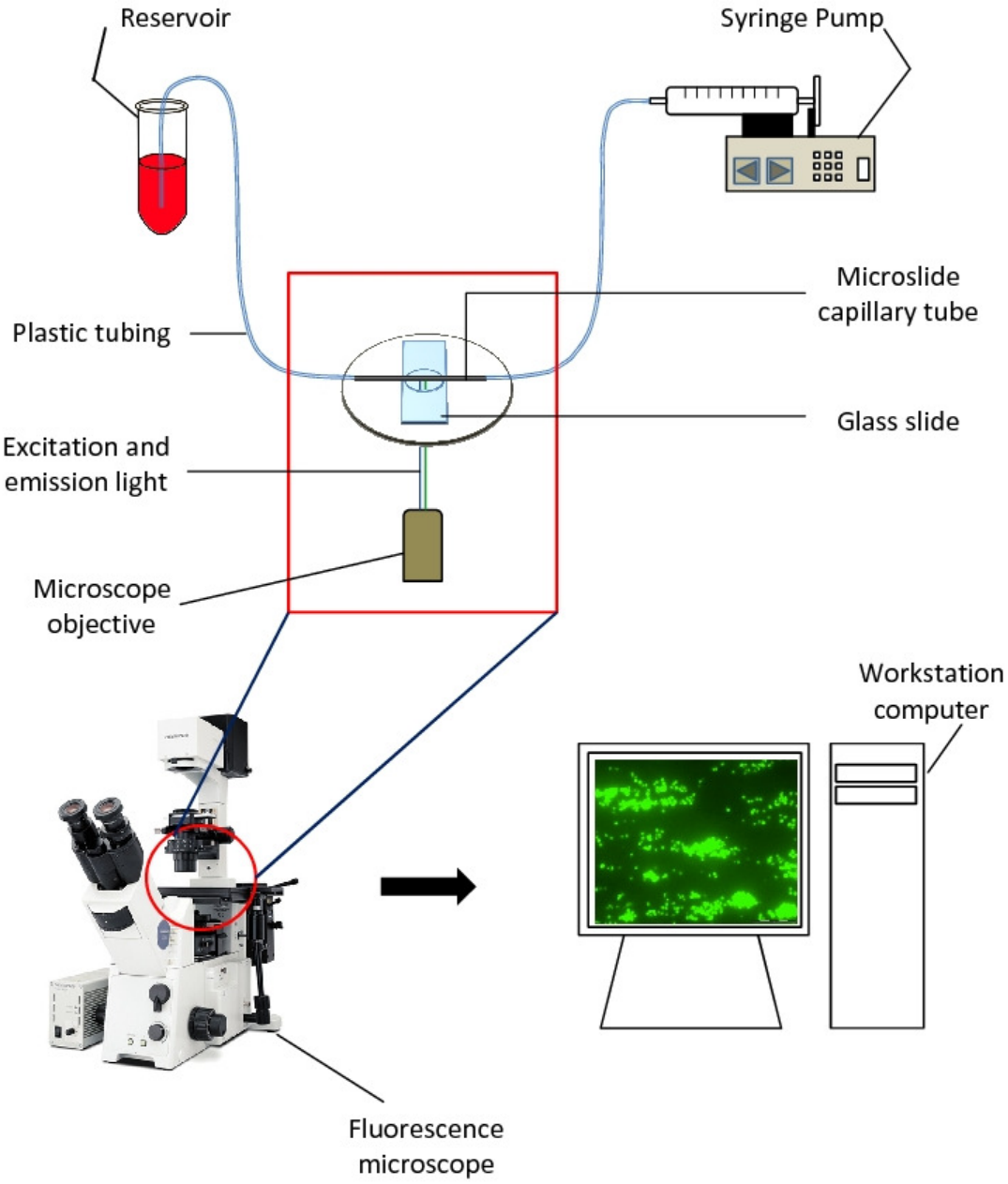


Figure 2.3: Set-up of equipment used to study platelet adhesion and thrombus formation under flow. Fluorescently-stained platelets in whole blood or reconstituted with autologous red blood cells were perfused through protein-coated microslide capillary tubes connected to a syringe pump by a series of plastic tubes. Images and videos of thrombus formation were captured using a fluorescence microscope fitted with a digital camera connected to a workstation computer. Microscope image from reference (Olympus, 2010).

In brief, washed platelets (4×10^8 platelets/mL) were incubated with $DIOC_6$ (1µM) at 37°C for 10min before reconstitution with autologous washed RBC to final concentrations of 2×10^8 platelets/mL and 50% (v/v) RBC. Cells were washed to exclude confounding effects of plasma proteins. Flow studies were performed using glass microslide capillary tubes coated with FXIII (200µg/mL) or FXIIIa (200µg/mL) for 12 hours and blocked with BSA/PBS (10mg/mL) for 1h at room temperature. Using a syringe pump, platelet/RBC suspension was drawn from a reservoir through coated tubes at desired shear rates for 4min, followed by washing with modified Tyrode's buffer for a further 4min at equivalent shear to remove unstably bound platelets (McCarty et al., 2005). The ability of platelets in whole blood treated with PPACK (40µM) to interact with immobilised FXIII was similarly assessed.

In order to quantify platelet arrest, real-time videos and images of stably adherent platelets/thrombi were recorded under the fluorescence microscope (×60 objective). ImageJ software was then used to analyse and process captured images. For each experimental condition, images of random view fields covering a total area of $0.1mm^2$ were used to calculate platelet surface area coverage (%), since the software could not fully discriminate between single platelets and platelet aggregates. To measure surface coverage, images were saved in 8-bit grayscale format. Adherent platelets appeared white against a black background. ImageJ software was used to calculate the specific areas covered by the white regions relative to the entire image, expressing the result as percentage area coverage. Measurement of thrombus volume was performed as previously described (Ross et al., 1995). Briefly, images were captured and stored in 8-bit grayscale format. Thrombus area values were then multiplied by their corresponding mean gray values which coincided with

fluorescence intensity. The product was regarded as thrombus volume and measured in arbitrary units (Ross et al., 1995, Savage et al., 1998, Matsui et al., 2002).

For some flow experiments, increasing FXIIIa concentrations (20–200μg/mL) were co-immobilised with vWF (20μg/mL) on the capillary tubes. To investigate how FXIIIa bound to collagen and influenced its ability to support platelet arrest, collagen (100μg/mL) was initially incubated with FXIIIa (200μg/mL) for 15min at 37^{o}C. The mixture was then used to coat capillary tubes. Platelet adhesion under flow, video or image capture and analysis were subsequently carried out as described above. In other experiments, platelets were pretreated with RGDS (500μM) to broadly inhibit platelet integrins, anti-$\alpha_v\beta_3$ (LM609, 20μg/mL) to block $\alpha_v\beta_3$, tirofiban (2μM) to specifically inhibit $\alpha_{IIb}\beta_3$ function or modified Tyrode's buffer as a control. Incubations were for 20 minutes at 37°C.

2.7 Measurement of aggregation and secretion in suspended platelets

Light-transmission aggregometry was developed by Born in 1962 and revolutionised the investigation of processes which initiate and influence platelet aggregation. The technique is based on changes in light scattering through a platelet suspension which is detected by a photocell. It is assumed that when using small volumes of platelet suspension with stirring, resting platelets are uniformly distributed. This forms an optically dense medium which is refractory to the passage of light. However, following their activation, platelets aggregate into clumps generating transparent gaps that allow light to pass through (Figure 2.4A). The amount of light transmission is proportional to the extent of platelet aggregation which is in turn dependent on the level of platelet activation (Figure 2.4B).

2.7.1 Light-transmission aggregometry

Aggregation was monitored using a Chrono-log dual-channel light aggregometer. Washed platelets (3×10^8 platelets/mL) were incubated at 37°C with stirring (1000rpm) for 1min to allow temperature equilibration and then stimulated with the required agonists for 3 minutes at 37°C. Percentage aggregation was recorded after 3 minutes from the aggregation traces produced by Aggro/Link computer software from Chrono-log.

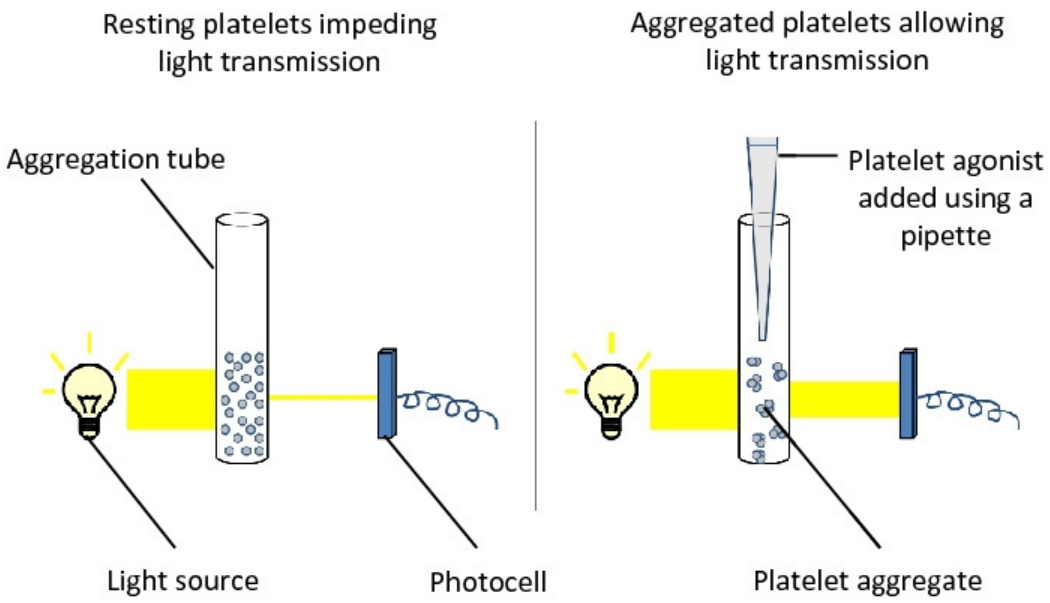
2.7.2 Lumi-aggregometry

ATP is an essential cofactor in the oxidation of luciferin to oxyluciferin catalysed by firefly luciferase. In the reaction, yellow light and AMP are given off as by-products. Hence, ATP

released from dense granules of activated platelets in the presence of Chronolume reagent (firefly luciferin/luciferase) leads to fluorescent-light generation which is detected by a luminescence (lumi)-aggregometer.

Platelet dense granule secretion was measured using a Chrono-log lumi-aggregometer. Chrono-lume reagent (2nM) was added to washed platelets (3×10^8 platelets/mL) followed by stirring (1000rpm) for 1min to allow temperature equilibration. Platelets were then stimulated with the desired agonist for 4min at 37°C under stirring conditions (1000rpm). Using ATP (1nM) as standard (100%) to calibrate the machine, the percentage platelet ATP secretion after 4 minutes was recorded from traces produced by Aggro/Link computer software from Chrono-log.

A)


Resting platelets impeding light transmission
Aggregated platelets allowing light transmission
Aggregation tube
Platelet agonist added using a pipette
Light source
Photocell
Platelet aggregate

B)

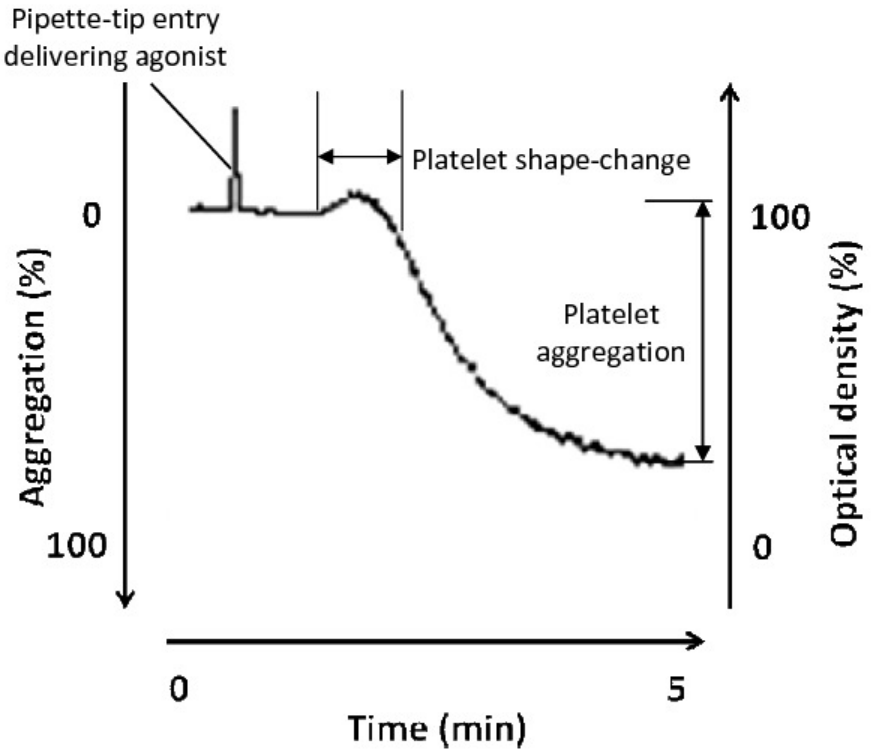

Pipette-tip entry delivering agonist
Platelet shape-change
0
100
Platelet aggregation
Aggregation (%)
100
0
Optical density (%)
0
5
Time (min)

Figure 2.4: Measurement of platelet aggregation using a light-transmission aggregometer. **(A)** Upon agonist stimulation, platelets aggregate, allowing increased transmission of incident light. This change is detected by a photocell in the aggregometer and quantified using specialist computer software. **(B)** A typical aggregation trace generated from measurements by the aggregometer software showing changes in optical density (platelet aggregation) relative to time.

2.8 Methods for the assessment of platelet signalling events

Platelet adhesion to certain exogenous proteins such as collagen and fibrinogen is known to trigger biochemical signal transduction resulting in activatory responses. Such signalling often involves sequential phosphorylation of intracellular proteins which may alter their functional states. To enable examination of individual cellular proteins, adherent platelet lysates can be separated using gel electrophoresis. The proteins can then be identified and further analysed through western blotting.

2.8.1 Gel electrophoresis

Electrophoresis is the movement of charged molecules such as proteins in an electric field. When an electric field is applied across a porous matrix such as a gel, it can be used to separate proteins which migrate through the gel to different extents depending on their molecular weight and charge. In polyacrylamide gel electrophoresis (PAGE), gels consisting of polymerised acrylamide are used to separate proteins by distinctive electrophoretic migration. Acrylamide molecules polymerise into long linear chains which are cross-linked by bis-acrylamide. This polymerisation is accelerated by the presence of free radicals which are generated by the addition of ammonium persulphate (APS). Tetramethylethylenediamine (TEMED) is also added to catalyse the generation of free radicals from APS (Shi and Jackowski, 1998). The percentage of the acrylamide used determines gel pore size and therefore the relative separation of proteins within a mixture.

Electrophoretic mobility is influenced by protein shape, charge-density and molecular weight. In order to separate proteins solely by molecular weight, sodium dodecyl sulphate (SDS) is added to the protein mixture (Shapiro et al., 1967). SDS is an anionic detergent that binds and denatures proteins leaving them with similar, rod-shaped tertiary structure. Furthermore, it confers equal negative charge per unit protein mass (1.4g SDS per 1g protein). In the presence of a reducing agent such as 2-mercaptoethanol, disulphide bonds are broken and proteins become fully denatured. Proteins treated this way are then separated by their differential migration through a polyacrylamide gel under an electric field (SDS-PAGE).

2.8.1.1 Sample preparation for SDS-PAGE

To investigate signalling events occurring in FXIII-adherent platelets; 6-well culture plates were coated with either HS (5%), FXIIIa (200µg/mL) or fibrinogen (200µg/mL) overnight at 4°C. Uncoated surfaces were blocked for 30min with HS (5%). Washed platelets (5×10^8 platelets/mL) were added to the wells in the presence of apyrase (1U/mL) and indomethacin (10µM) to preclude signalling events downstream of secreted ADP and TXA_2 respectively. Platelets were allowed to adhere for 45min at 37°C. For each investigated parameter, duplicate wells were prepared in order to increase the yield of adherent platelets. Non-adherent platelets were removed by 2 washes with PBS and adherent platelets lysed with ice-cold Laemmli buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 50mM trizma base, bromophenol blue (trace), pH 6.8). Lysates were scraped from the wells using a rubber policeman, transferred to eppendorfs and left on ice for 30min. They were then centrifuged at 8500*g* for 5min to pellet insoluble debris. The supernatant was separated and retained in

fresh eppendorfs. Aliquots were withdrawn to measure protein concentration (section 2.8.1.2).

2.8.1.2 Protein Assay

To facilitate equal protein loading of samples during electrophoresis, their protein concentrations were quantified using a Bio-Rad detergent compatible (DC) protein assay kit (Bio-Rad, UK). The principle of the assay stems from reactions occurring between proteins in alkaline copper tartrate solution and Folin reagent. Firstly, the proteins reduce copper in an alkaline medium which then reduces Folin reagent turning it from yellow to blue in colour with maximal light absorption at 750nm (Lowry et al., 1951). The change in light absorption can then be detected using a plate reader or a spectrophotometer.

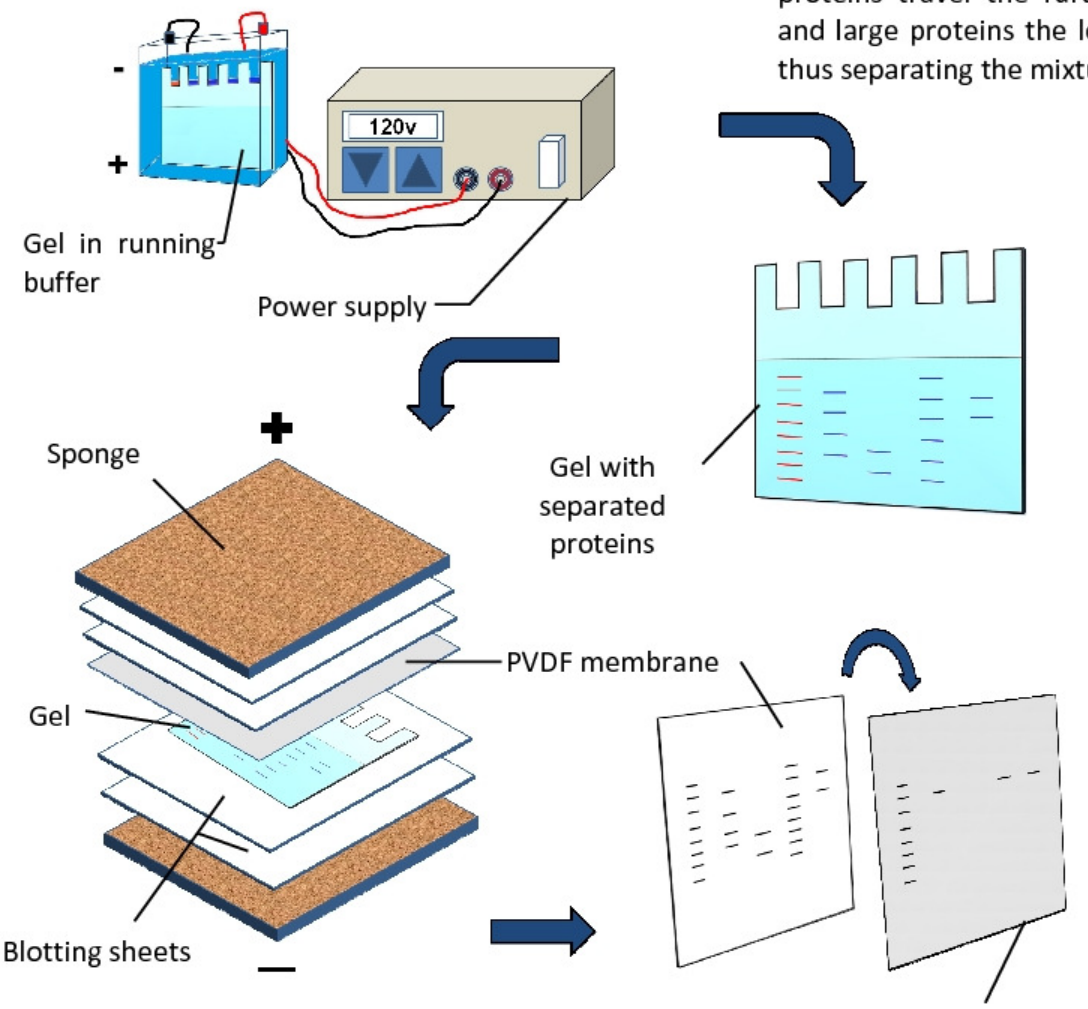
BSA was used as protein standard and 5 dilutions (0.3, 0.6, 0.9, 1.2 and 1.5mg/mL) were prepared in modified Laemmli buffer to measure protein concentrations of whole-cell lysates or immunoprecipitation lysis buffer to measure that of immunoprecipitates. Samples and protein standards were added to the wells of a microplate in triplicate. This was followed by addition of copper tartate solution and Folin reagent. The microplate was then placed on a shaker for 15 minutes. Light absorption at 750nm was determined using a plate reader. Absorption values from the protein standards were used to plot a standard curve from which protein concentrations of the samples were derived by interpolation.

2.8.1.3 Methodology used for SDS-PAGE

All samples were run under discontinuous SDS-PAGE under reducing conditions according to the method of Laemmli (1970) (Figure 2.5; points 1 and 2). 1.5mm thick polyacrylamide gels comprising 4% acrylamide stacking gels (pH 6.8) overlying 10-18% gradient acrylamide resolving gels (pH 8.8) were prepared to separate proteins (Table 1). The stacking gel contained wells for protein loading and 30µg of sample protein were loaded in each well. Biotinylated and prestained protein molecular weight markers were also added alongside the samples. After running buffer (25mM trizma base, 192mM glycine, 0.1% w/v SDS, pH 8.3) had been added to a gel tank, gels were left to run at a constant voltage (120V) for 2.5 hours. Under these conditions, proteins had net negative charges and migrated downwards toward the anode.

1. Proteins are loaded into the stacking gel and electric current applied for SDS-PAGE. Due to the alkaline running buffer, they migrate to the anode.

2. In the resolving gel, small proteins travel the furthest and large proteins the least, thus separating the mixture.



3. Proteins are transferred from the gel to a PVDF membrane in alkaline buffer under an electric field; again moving towards the anode.

4. The PVDF membrane is probed with protein-specific primary antibodies. Subsequent incubation with HRP-linked secondary antibodies allows exposure of photographic films by ECL.

Figure 2.5: Outline of the procedures involved in separating proteins by SDS-PAGE and Western blotting. SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; ECL, enhanced chemiluminescence; HRP, horse-radish peroxidise.

Table 1: Composition of polyacrylamide gels used for electrophoretic protein separation

	4% stacking gel	10% resolving gel	18% resolving gel
Deionised water	4.87mL	1.418mL	0,708mL
Acrylamide (30%)	0.75mL	1.182mL	1.961mL
Buffer I *	-	0.886mL	0.886mL
Buffer II **	1.87mL	-	-
Ammonium persulphate (10%)	75µL	18µL	18µL
Tetramethylethyl-enediamine	10µl	2µl	2µl

* Buffer I: 1.5M trizma base, 0.4% w/v sodium dodecyl sulphate, pH 8.8

** Buffer II: 0.5M trizma base, 0.4% w/v sodium dodecyl sulphate, pH 6.8

2.8.2 Immunoprecipitation

Immunoprecipitation is a method used to isolate proteins from a mixture such as a cell extract (Figure 2.6). An antibody selective for a target protein is added to the mixture to form antibody-antigen complexes. The complexes are then precipitated by adsorbing the antibodies to an insoluble matrix such as agarose or sepharose beads conjugated to protein A or G. The latter two proteins derive from bacterial walls and are stably bound by antibody Fc regions. Using centrifugation, the beads are pelleted and the supernatant with unwanted proteins is aspirated and discarded. After several washes, the antibody-antigen complex is liberated from the beads by boiling in Laemmli buffer (Rosenberg, 2005). The isolated protein may be further studied through electrophoresis and western blotting (sections 2.8.1 and 2.8.3 respectively). In addition to concentrating proteins that would otherwise be too scant to detect by ordinary western blotting, immunoprecipitation enables biochemical analysis of single, purified proteins.

2.8.2.1 Methodology used in immunoprecipitation

Platelets were adhered to protein-coated 6-well plates as outlined in section 2.8.1.1. Adherent platelets were lysed with immunoprecipitation lysis buffer (150mM NaCl, 10mM Tris, 1mM EDTA, 1mM EGTA, 1% Igepal, 1mM PMSF, 5µg/mL leupeptin, 5µg/mL aprotinin, 0.5µg/mL pepstatin, 2.5mM Na_3VO_4, pH 7.4). Syk, LAT, PLCγ2 and SLP-76 were subsequently immunoprecipitated as previously described (Riba et al., 2005). Briefly, a suspension of either protein A or G sepharose beads (50%, w/v) in TBS-T was prepared (slurry). Equal aliquots of slurry were distributed in the required number of eppendorfs. For each target protein, a

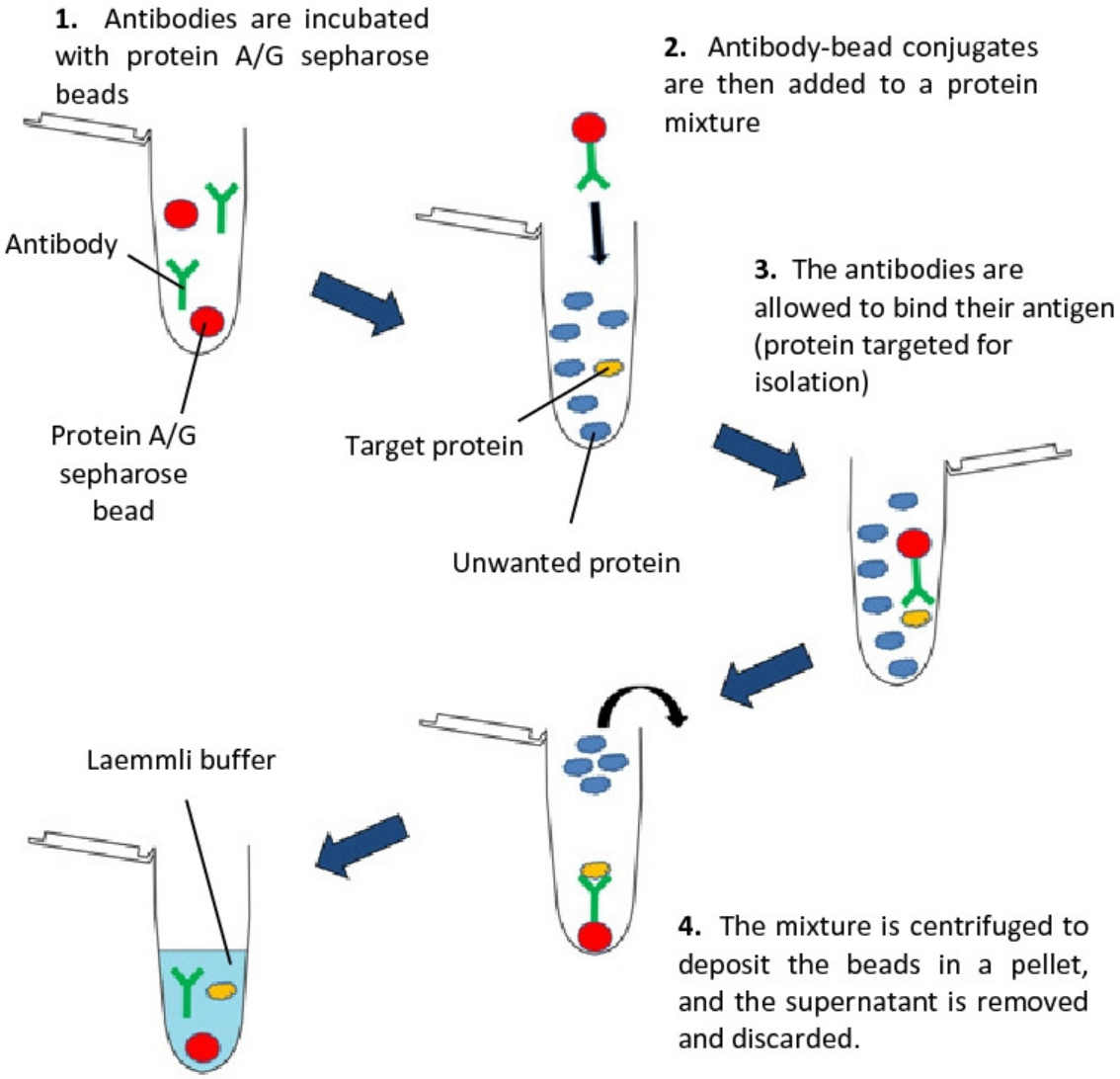


Figure 2.6: Summary of principles involved in isolating a protein from a mixture by immunoprecipitation.

predetermined optimal amount of selective antibody was then added to the slurry. An isotype-matched control IgG antibody was used to control against non-specific antibody-protein interactions. The mixture was incubated with constant agitation for 1hr at 4°C to allow antibodies to bind to the beads.

In parallel, lysates were precleared by incubation with aliquots of slurry for 1hr at 4°C with agitation. Precleared lysates were centrifuged at 8500*g* for 1min to pellet the beads and any insoluble cell debris. The supernatant was subsequently separated and equally dispensed into the eppendorfs containing antibody-bound beads.

The mixture was agitated overnight at 4°C to allow antibodies to bind their target proteins. It was then centrifuged at 8500*g* for 1min to isolate the beads. The supernatant was removed and beads washed sequentially, once with lysis buffer and twice with TBS. After these washes to remove any proteins remaining from the supernatant, the beads were boiled in Laemmli buffer to liberate the antibodies and target proteins. Finally, the isolated proteins were separated by SDS-PAGE (section 2.8.1.3) and further analysed by western blotting (section 2.8.3.1).

2.8.3 Western blotting

To identify specific proteins separated by SDS-PAGE, western blotting is routinely used. This involves transferring proteins from a gel to an adhesive matrix such as nitrocellulose or polyvinylidene fluoride (PVDF) membranes under an electric field. Once transferred, the membranes are probed with specific primary antibodies against the targeted proteins. A

secondary antibody raised against the primary antibody species is then added. Secondary antibodies are commonly conjugated to horseradish peroxidise (HRP) which allows the detection of their position on the membrane through exposure of photographic film by enhanced chemiluminescence (ECL). ECL is the emission of light caused by oxidation of luminol by hydrogen peroxide, catalysed by HRP and perpetuated by an "enhancer" such as *p*-coumaric acid (Thorpe et al., 1985, Haan and Behrmann, 2007). The principal methodology involved in SDS-PAGE and western blotting is summarised in Figure 2.5.

2.8.3.1 Methodology used in western blotting

Proteins separated by SDS-PAGE (section 2.8.1.3) were transferred to PVDF membranes at constant voltage (100V) for 2.5hrs. This involved arranging the gel and membrane in a transfer cassette immersed in alkaline transfer buffer (25mM trizma base, 192mM glycine, 20% v/v methanol, pH 8.3) as shown schematically in Figure 2.5 (point 3). Under these conditions, proteins migrating towards the anode were captured by the PVDF membrane. The membranes were blocked with BSA/TBS-T (10%, w/v) for 1hr to prevent non-specific antibody binding and subsequently probed with an anti-phosphotyrosine monoclonal antibody (1:1000 in 2% BSA/TBS-T) overnight at 4°C. The membranes were rinsed with TBS-T for 10 minutes, probed with HRP-conjugated secondary antibodies; anti-rabbit or anti-mouse (both 1:10000) and anti-biotin (1:2000) for 1 hr, washed with TBS-T (4 washes, 15 minutes each). Membranes were incubated with enhanced chemiluminescence (ECL) solutions for 90s and used to expose photographic film for times ranging from 5s to 10min.

In some cases, membranes were subsequently stripped by agitated incubation with Restore™ western blot stripping buffer (Thermo Scientific, UK) for 20min at room temperature followed by two 10min washes with TBS-T as per manufacturer's instructions. After blocking with BSA/TBS-T (10%, w/v) for 30min, the membranes were reprobed overnight with anti-β-tubulin (1:1000) to assess equal loading of proteins in each lane. For immunoprecipitation experiments (2.8.2.1), equal protein loading was assessed using the same antibodies that had been used to immunoprecipitate each protein. Namely, these were: anti-Syk (1:1000), anti-LAT (1:1000), anti-PLCγ_2 (1:1000), anti-SLP-76 (1:1000) or anti-FXIII-A (1:1000).

2.9 Statistical analysis

Results are expressed as means ± standard deviation (SD) and were analysed using the Student's *t*-test or analysis of variance (ANOVA) as described in the relevant sections. Statistical significance was taken at levels $P<0.05$ and $P<0.01$. Statistical tests were applied using Microsoft Office Excel (2007) and the Statistical Package for Social Sciences (SPSS, version 15).

CHAPTER 3 - IMMOBILISED FXIII SUPPORTS PLATELET ADHESION AND SPREADING

3.1 Introduction

Physiologically, platelets adhere to suspended proteins such as fibrinogen as well as immobilised proteins like collagen (Ruggeri and Mendolicchio, 2007, Rivera et al., 2009). Although numerous studies have investigated platelet binding to FXIII in suspension (Greenberg and Shuman, 1984, Kreager et al., 1988, Cox and Devine, 1994, Nagy et al., 2009), to date only a single unconfirmed study has explored these interactions with the immobilised enzyme (Dardik et al., 2002b). Findings from this study suggested that both surface-immobilised FXIII and FXIIIa support platelet adhesion. However, the independence of the adhesive interaction from the enzymatic activity of FXIIIa was not definitively shown nor was contamination of the plasma FXIII preparation used with other platelet adhesive proteins strictly attested. Moreover, the study did not examine whether physiological platelet responses followed adhesion to FXIII, in particular, whether adherent platelets became activated and spread.

Characterisation of platelet interactions with immobilised FXIII is of interest because the enzyme may localise on the ECM (Blanchy et al., 1986, Kikuchi et al., 1986, Gautheron et al., 1988), where it cross-links a number of matrix proteins (Mosher, 1984, Bockenstedt et al., 1986). This reorganisation of the ECM facilitates cell attachment and migration, thereby

explaining the significant involvement of FXIII in wound healing (Biel et al., 1971, Cario et al., 1999, Inbal et al., 2005).

Aims:

The experiments described in this chapter were designed to characterise the nature of platelet adhesion to immobilised FXIII. More specifically, the objectives were to:

- Establish whether platelets can adhere to immobilised FXIII independently of other proteins.
- Investigate the importance of FXIII enzymatic activity in supporting platelet adhesion.
- Determine whether FXIII can stimulate platelet spreading.

3.2 Absence of contaminating fibrinogen from Fibrogammin P

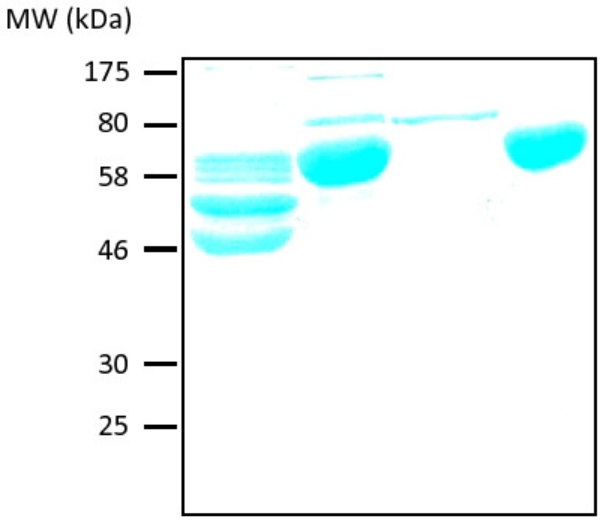
Human FXIII used throughout the study was derived from Fibrogammin P; a commercial purified and concentrated plasma FXIII preparation. Since plasma FXIII circulates bound to fibrinogen (Greenberg and Shuman, 1982, Siebenlist et al., 1996), which is an established platelet ligand, it was necessary to test the purity of Fibrogammin P before performing platelet adhesion experiments. This was achieved by applying two different methods.

Proteins present in Fibrogammin P were separated by SDS-PAGE and stained with Coomassie brilliant blue to reveal their positions within the gel. Fibrogammin P separated into 3 distinct bands under SDS-PAGE (Figure 3.1A, lane 2). The uppermost band had a molecular weight

corresponding to FXIII-A_2 and FXIII-B_2 dimers (approximately 160kDa). The band beneath it appeared at the characteristic molecular weight of monomeric FXIII-A and FXIII-B under denaturing SDS-PAGE (approximately 80kDa) (Schwartz et al., 1971). According to the manufacturer, albumin is the only other protein present in Fibrogammin P apart from FXIII (Electronic Medicines Compendium, 2010). Consistent with this, the third observable band appeared at the molecular weight of albumin (67kDa; Figure 3.1A, lane 2). To aid with identification of proteins, rFXIII-A, fibrinogen and BSA were also loaded into separate wells on the same gel. Fibrinogen separated into its three constituent chains (Aα, Bβ and γ) under SDS-PAGE (Figure 3.1A; lane 1). BSA was also used as a negative control for fibrinogen and only one protein band (67kDa) was recognisable (Figure 3.1A, lane 4). Importantly, all three fibrinogen chains were visibly absent from rFXIII-A (Figure 3.1A, lane 3). Although albumin masked the position of fibrinogen Aα chains which have a comparable molecular weight (64kDa), Bβ and γ chains could not be seen, strongly suggesting the absence of fibrinogen from Fibrogammin P (Figure 3.1A, lane 2).

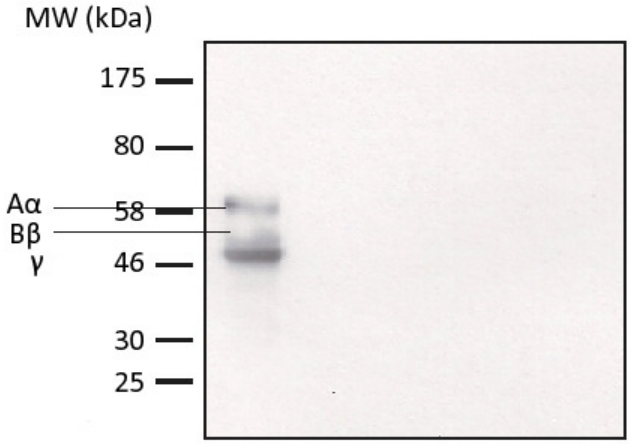
Western blotting was used as secondary methodology to confirm the absence of fibrinogen from Fibrogammin P. After separation of fibrinogen, Fibrogammin P, rFXIII-A and BSA by SDS-PAGE, they were transferred to PVDF membranes which were probed with a polyclonal anti-fibrinogen antibody (Figure 3.1B). The antibody detected all three fibrinogen chains in the positive control (Figure 3.1B, lane 1). However, none could be detected in the BSA negative control (Figure 3.1B, lane 4), Fibrogammin P (Figure 3.1B, lane 2) or rFXIII-A (Figure 3.1B, lane 3).

A)



Fibrinogen	+	-	-	-
Fibrogammin P	-	+	-	-
rFXIII-A	-	-	+	-
BSA	-	-	-	+
Lane	1	2	3	4

B)



IB: fibrinogen

Fibrinogen	+	-	-	-
Fibrogammin P	-	+	-	-
rFXIII-A	-	-	+	-
BSA	-	-	-	+
Lane	1	2	3	4

Figure 3.1: The purity of Fibrogammin P and recombinant FXIII-A as assessed by SDS-PAGE and immunoblotting. The purities of Fibrogammin P and recombinant FXIII-A (rFXIII-A) were assessed by gel electrophoresis and immunoblotting. Fibrinogen, Fibrogammin P, rFXIII-A and bovine serum albumin (BSA) were loaded onto a 10-18% gradient polyacrylamide gel (20µg of protein/well except for rFXIII-A: 4µg/well). Electrophoresis was then carried out for 2.5h at 120V under reducing conditions. **(A)** Proteins in the gel were stained with Coomassie blue staining solution before being photographed. **(B)** Proteins were transferred to polyvinylidene fluoride (PVDF) membranes for 2.5h at 100V, and immunoblotted with a polyclonal anti-fibrinogen antibody overnight at 4°C. Membranes were then incubated with a HRP-conjugated secondary antibody for 1h at room temperature and used to expose photographic film by enhanced chemiluminescence (ECL). Blots are representative of 4 independent experiments with the individual batches of Fibrogammin P used in this study. Labelled in lane 1 are the 3 constituent chains of fibrinogen. MW: molecular weight. IB: Immunoblot.

3.3 FXIII-A is present in Fibrogammin P and & rFXIII-A

Having ensured the absence of contaminating fibrinogen, the presence of FXIII-A in Fibrogammin P was tested by immunoblotting using a FXIII-A specific monoclonal antibody. The antibody detected a distinct band in Fibrogammin P at the expected molecular weight of FXIII-A (83kDa) (Figure 3.2A, lane 1). The antibody also detected a band of identical molecular weight in a lane loaded with rFXIII-A (Figure 3.2A, lane 2), but failed to bind in a BSA negative control (Figure 3.2A, lane 3). Thus, FXIII-A was present in both Fibrogammin P and the recombinant FXIII-A preparation. Moreover, human and recombinant FXIII-A were indistinguishable in terms of molecular weight.

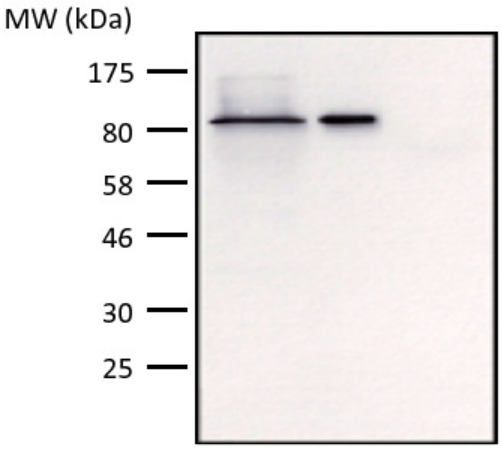
Since human serum was to be used as a blocking agent, it was also analysed for FXIII-A content by western blotting using a monoclonal antibody specific for FXIII-A subunits. In contrast to Fibrogammin P which displayed a distinct band representing FXIII-A (Figure 3.2B, lane 1), none was detected in the human serum preparation (Figure 3.2B, lane 2). The human serum was therefore considered free from FXIII-A, and was not expected to influence analyses of FXIII-mediated platelet adhesion.

3.4 Enzymatic activation of FXIII by thrombin *in vitro*

Fibrogammin P (FXIII) and rFXIII were incubated with thrombin (10U/ml) in the presence of calcium (10mM) and transglutaminase activity was monitored by measuring cross-linking of

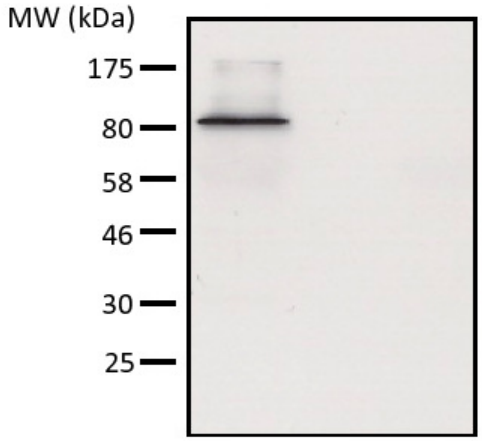
biotinylated pentylamine to immobilised fibrinogen using the method of Song (1994) (section 2.3.2) without further addition of thrombin and calcium. Under this method, FXIIIa and rFXIIIa both induced significantly increased absorption values of 1.331 (P<0.01 vs FXIII) and 1.318 (P<0.01 vs rFXIII) compared to their respective controls. Importantly, both appeared to have similar specific activity as there was negligible difference in their absorption values (P>0.05). In contrast FXIII and rFXIII induced no cross-linking as evidenced by minimal light absorption values of 0.105 and 0.101 respectively (P>0.05) (Figure 3.3). Similarly, catalase, which was used as a negative control for transglutaminase activity, also gave a low absorption value of 0.100 (P>0.05 vs FXIII and rFXIII). These data, show that FXIIIa and rFXIIIa acquired transglutaminase activity under the conditions used to activate them.

A)



Fibrogammin P	+	-	-
rFXIII-A	-	+	-
BSA	-	-	+
Lane	1	2	3

B)



Fibrogammin P	+	-	-
Human Serum	-	+	-
BSA	-	-	+
Lane	1	2	3

Figure 3.2: FXIII-A subunits are present in Fibrogammin P and recombinant FXIII-A, but not human serum. (A) Fibrogammin P, recombinant FXIII-A (rFXIII-A) and bovine serum albumin (BSA) were loaded onto a 10-18% gradient polyacrylamide gel (20µg of protein/well except for rFXIII-A: 4µg/well). Electrophoresis was then carried out for 2.5h at 120V under reducing conditions. Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes for 2.5h at 100V, and probed with a monoclonal anti-FXIII-A antibody overnight at 4°C. Membranes were then incubated with a HRP-conjugated secondary antibody for 1h at room temperature and used to expose photographic film by enhanced chemiluminescence (ECL). The blot is representative of those from 4 independent experiments with the individual batches of Fibrogammin P used in this study. (B) As in (A) except human serum (20µg of protein/well) was also loaded in the gel. MW: molecular weight. IB: Immunoblot.

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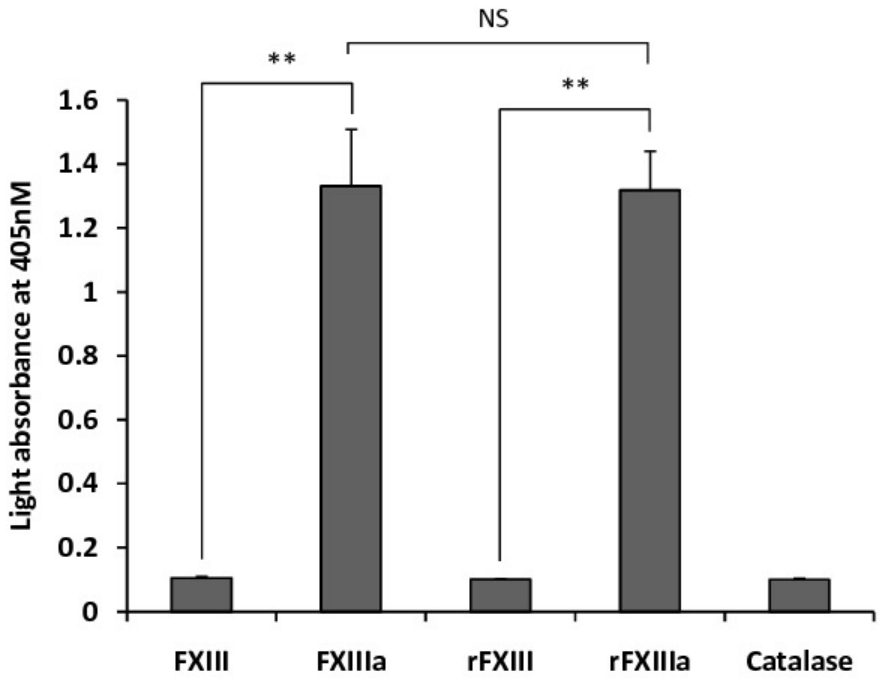


Figure 3.3: Factor XIII acquires transglutaminase activity following activation *in vitro*. Plasma factor XIII (FXIII, 200µg/mL) and recombinant human factor XIII-A (rFXIII, 200µg/mL) were incubated with thrombin (10U/mL) in the presence of calcium (10mM) for 1h at 37°C. Thrombin was then inactivated by addition of Phe-Pro-Arg-Chloromethylketone (PPACK, 20µM) for 10min at 37°C. Transglutaminase activity was measured by amine incorporation into fibrinogen as described by Song et al., (1994). Briefly, wells of a 96-well microplate were coated with fibrinogen (40µg/mL) for 45min and uncoated surfaces blocked with BSA/TBS (1%) for 1h at room temperature. The following were then added at the indicated final reactive concentrations: FXIII, FXIIIa, rFXIII, rFXIIIa and catalase (all 22µg/mL), 5-(biotinamido)pentylamine (1mM) and dithiothreitol (0.5mM) and left for 10min. Streptavidine-alkaline phosphatase (1µg/mL in 0.1% BSA/TBS) was added for 1h to conjugate specifically with fibrinogen-bound 5-(biotinamido)pentylamine then the wells were incubated with *p*-nitrophenylphosphate for 30min at 37°C. NaOH (4M) was subsequently added to stop colour development and optical density measured at 405nm using a microplate reader. Data shown are mean ± SD of 3 independent experiments. **: $P<0.01$.

3.5 PPACK eliminates thrombin reactivity with platelets

Since FXIII was activated by thrombin, a potent platelet agonist (Crawley et al., 2007), it was important to establish that any residual platelet activating potential of thrombin was completely eliminated by PPACK as previously described (Dardik et al., 2002b). PPACK (20μM) eliminated thrombin-stimulated platelet aggregation when applied to thrombin (10U/mL) separately or within FXIIIa (200μg/mL) (Figure 3.4A). Moreover, when PPACK-treated thrombin was immobilised on glass slides it failed to support any platelet adhesion beyond that of human serum used to block platelet adhesion to uncoated glass (P>0.05) (Figure 3.4B). This was consistent with a previous report where PPACK inhibited thrombin activity and its ability to support platelet adhesion (Weeterings et al., 2006).

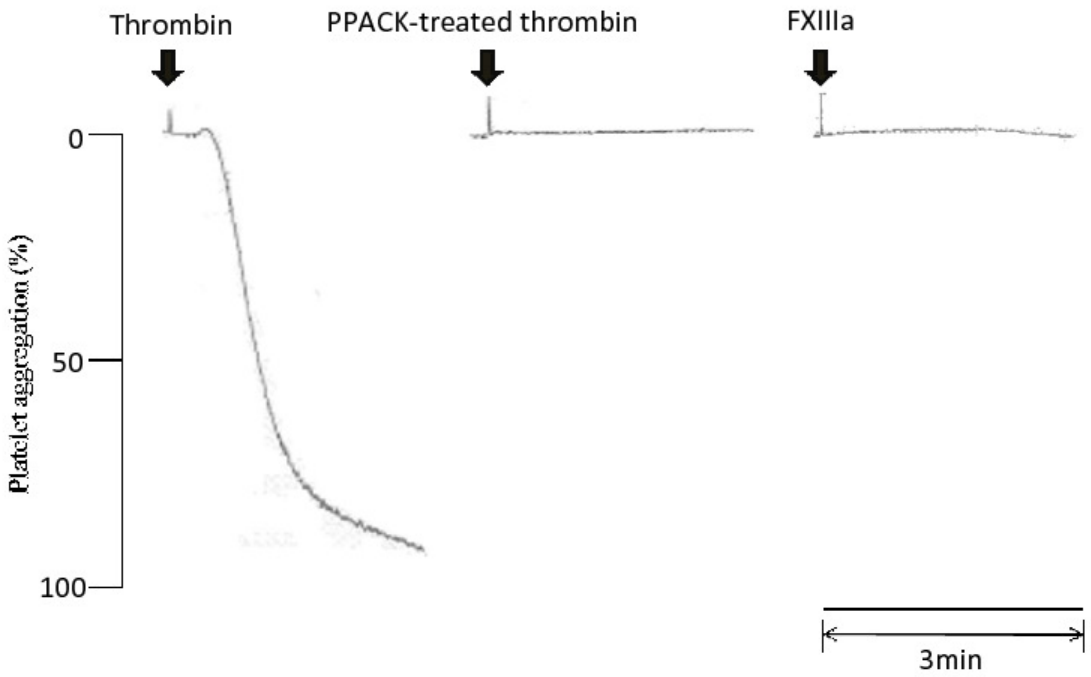
3.6 Platelets adhere to FXIII and FXIIIa under static conditions

3.6.1 FXIII and FXIIIa support similar levels of platelet adhesion

The ability of immobilised native and activated FXIII to support platelet adhesion was examined by a colorimetric assay (Bellavite et al., 1994) Washed platelets (1×10^8 platelets/mL) were adhered to either FXIII or FXIIIa (both 200μg/mL) immobilised on the wells of a microplate. Using this methodology, platelets were found to bind to both forms of enzyme to a similar extent, with absorption values of 0.92±0.10 for FXIII compared to 0.91±0.13 for FXIIIa (P>0.05) (Figure 3.5). In comparison, fibrinogen (200μg/mL) supported a significantly greater level of platelet adhesion as evidenced by an absorption value of

1.621±0.154 (P<0.05 vs FXIII and FXIIIa). As previously described, human serum supported negligible platelet adhesion (Maxwell et al., 2007), giving an absorption value of 0.208±0.012 (P<0.01 vs FXIII and FXIIIa, ANOVA).

A)


Thrombin
PPACK-treated thrombin
FXIIIa
0
50
100
Platelet aggregation (%)
3min

B)

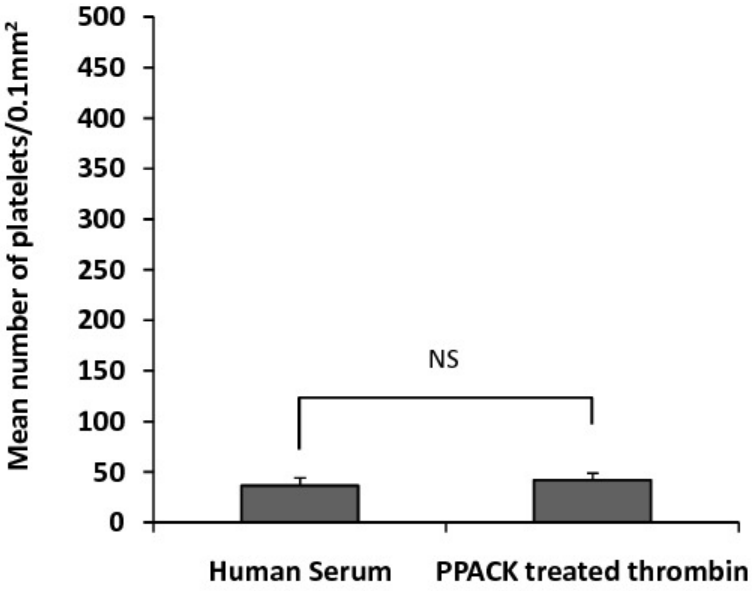

Mean number of platelets/0.1mm²
500
450
400
350
300
250
200
150
100
50
0
NS
Human Serum
PPACK treated thrombin

Figure 3.4: PPACK-treated thrombin neither stimulates platelets nor supports their adhesion. **(A)** Thrombin (10U/mL) was used to stimulate aggregation of washed platelets (3×10^8 platelets/mL) for 3min at 37°C under constant stirring (1000rpm). Platelet aggregation was measured using a Chrono-log dual channel light-transmission aggregometer and aggregation traces were generated by Aggro/Link computer software (Chrono-log, USA). In some cases thrombin was pretreated with Phe-Pro-Arg-Chloromethylketone (PPACK, 20μM) for 10min at 37°C. FXIIIa containing thrombin (10U/mL) which had been similarly inhibited by PPACK was also assessed for its ability to stimulate platelet aggregation. Shown are representative traces of 3 independent experiments with separate blood donors. **(B)** Glass slides were coated overnight with human serum or thrombin (10U/mL) which had been incubated with PPACK (20μM) for 10min at 37°C. Uncoated surfaces were blocked by overlay with human serum (5%) for 30min at room temperature. Washed platelets (5×10^7 platelets/mL) were then allowed to adhere to each surface for 1 hour at 37°C. Adherent platelets were stained with TRITC-phalloidin and viewed under the ×60 objective of an IX71 fluorescent microscope (Olympus, Japan). Platelets from random visual fields with a total area of $0.1mm^2$ were counted. Data shown are means ± SD and are from 3 independent experiments with separate blood donors. NS: not significant.

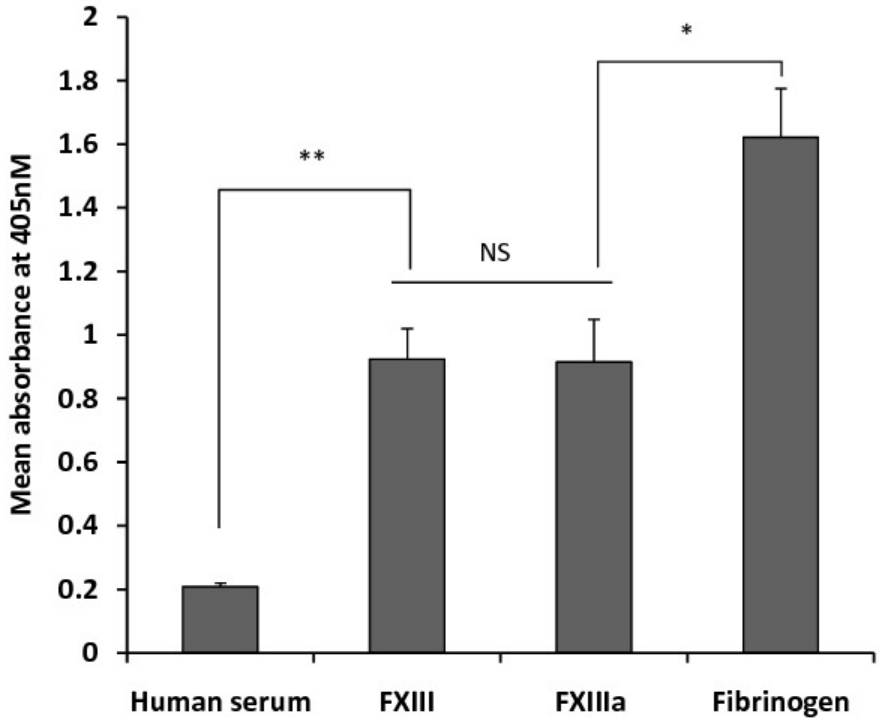


Figure 3.5: Platelets adhere equally to immobilised FXIII and FXIIIa as measured by the microplate method of Bellavite et al., (1994). Wells of a 96-well microplate were coated overnight with inactive or activated factor XIII (FXIII and FXIIIa respectively, 200µg/mL), human serum (5%) or fibrinogen (200µg/mL). Uncoated surfaces were blocked by overlay with human serum (5%) for 30min at room temperature. Washed platelets (1×10^8 platelets/mL) were then allowed to adhere to each surface for 1 hour at 37°C. Adherent platelets were incubated with reaction buffer (31mM citric acid, 5mM sodium citrate dehydrate, 5mM *p*-nitrophenylphosphate, 0.1% (v/v) Triton X-100, pH 5.4) for 1 hour at room temperature. The reaction was stopped by addition of NaOH (2M) to each well and optical density measured at 405nm. Data shown are means ± SD from 4 independent experiments with separate blood donors. **: $P<0.01$, *: $P<0.05$, NS: not significant.

The colorimetric assay suggested that FXIII and FXIIIa supported platelet adhesion equally, but it was important to confirm these findings by an independent method and perform a visual analysis to ensure that the increased absorption was due to adhesion and not aggregation or agglutination. Platelets were therefore adhered to wells on glass microscope slides coated with FXIII and FXIIIa (both adjusted to 200µg/mL) (section 2.5.2.1). FXIII(a) at a concentration of 200µg/mL (10U/mL) has previously been shown to support quantifiable platelet adhesion under static conditions (Dardik et al., 2002b). In addition, washed platelets were used at a concentration of 5×10^7 platelets/mL which allowed them to adhere as a monolayer of single cells that could be counted separately under this methodology (Hagmann, 1993). Both FXIII and FXIIIa supported platelet adhesion when they were immobilised on the slides. Similar numbers of adherent platelets were observed on each surface with 783±108 platelets/0.1mm^2 for FXIII compared to 713±82 platelets/0.1mm^2 for FXIIIa (P>0.05), suggesting equal platelet binding capacities (Figure 3.6). This was reproducible with native and activated forms of the recombinant enzyme with 548±92 platelets/0.1mm^2 adhering on rFXIII compared to 608±155 platelets/0.1mm^2 for rFXIIIa (P>0.05). Platelets also adhered readily to slides coated with fibrinogen (200µg/mL) which was used as a positive control. In fact, fibrinogen supported 1711±233 platelets/0.1mm^2, significantly more than FXIII and FXIIIa (P<0.05, ANOVA). Importantly, negligible platelet-binding (33±12 platelets/0.1mm^2) occurred on slides coated with human serum as a negative control.

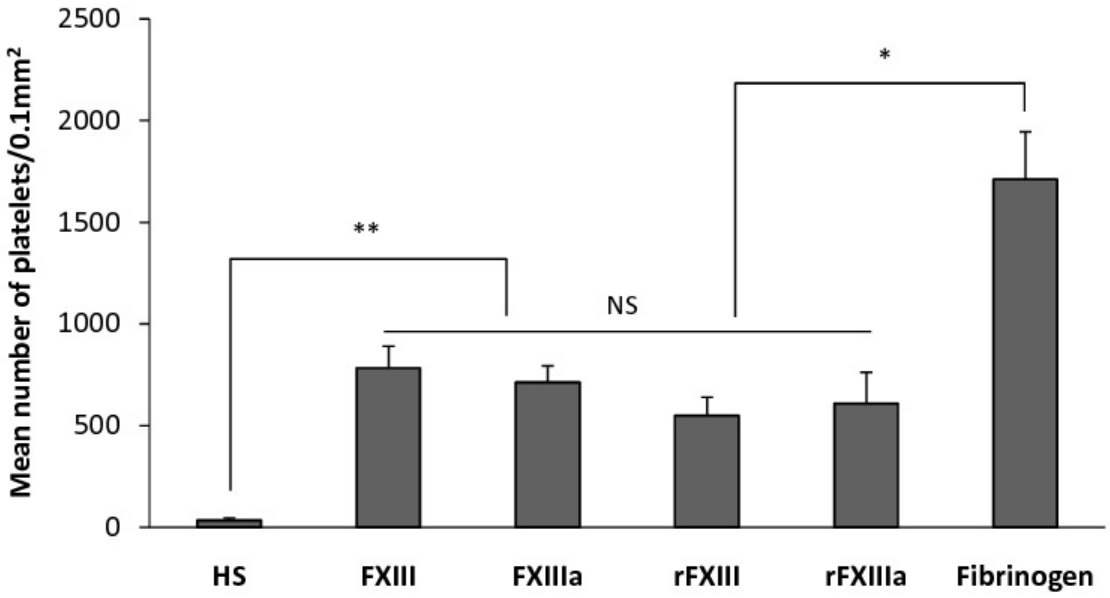


Figure 3.6: Platelets adhere similarly to FXIII and FXIIIa immobilised on glass slides as measured by fluorescence microscopy. Glass slides were coated overnight with inactive or activated factor XIII (FXIII and FXIIIa respectively, 200µg/mL), inactive or activated recombinant factor XIII-A (rFXIII and rFXIIIa respectively, 200µg/mL) or fibrinogen (200µg/mL). Uncoated surfaces were blocked by overlay with human serum (HS, 5%) for 30min at room temperature. Washed platelets (5×10^7 platelets/mL) were then allowed to adhere to each surface for 1h at 37°C. Adherent platelets were stained with TRITC-phalloidin and viewed under the ×60 objective of an IX71 fluorescent microscope (Olympus, Japan). Platelets from random visual fields with a total area of $0.1mm^2$ were counted. Data shown are means ± standard deviation and are from 4 independent experiments with separate blood donors.

3.6.2 Adherent platelets spread on immobilised FXIII and FXIIIa

Platelet adhesion to immobilised reactive proteins stimulates spreading responses (Behnke and Bray, 1988). To examine if this occurred with FXIII, washed platelets adherent on slides coated with either FXIII or FXIIIa (both 200µg/mL) were stained for filamentous actin (F-actin) using TRITC-phalloidin (section 2.5.2.1). This allowed observation of their actin-based cytoskeleton using fluorescence microscopy. Platelets adherent on both surfaces showed activatory morphological changes including filopodia extension and lamellipodia formation (Figure 3.7A). After 1 hour, most platelets were fully spread, displaying actin stress fibres. FXIII and FXIIIa stimulated platelet spreading to similar extents giving platelet surface areas of 24±5 and 28±3µm^2 (P>0.05) respectively. Moreover, this was comparable to spreading occurring on rFXIII, rFXIIIa and fibrinogen (all 200µg/mL) where platelet surface areas were 25±5, 26±3 and 24±1µm^2 respectively (P>0.05, ANOVA) (Figure 3.7B). Thus, in addition to supporting comparable levels of platelet adhesion, both FXIII and FXIIIa activated adherent platelets to induce spreading.

3.6.3 The influence of FXIII and FXIIIa concentration on platelet adhesion

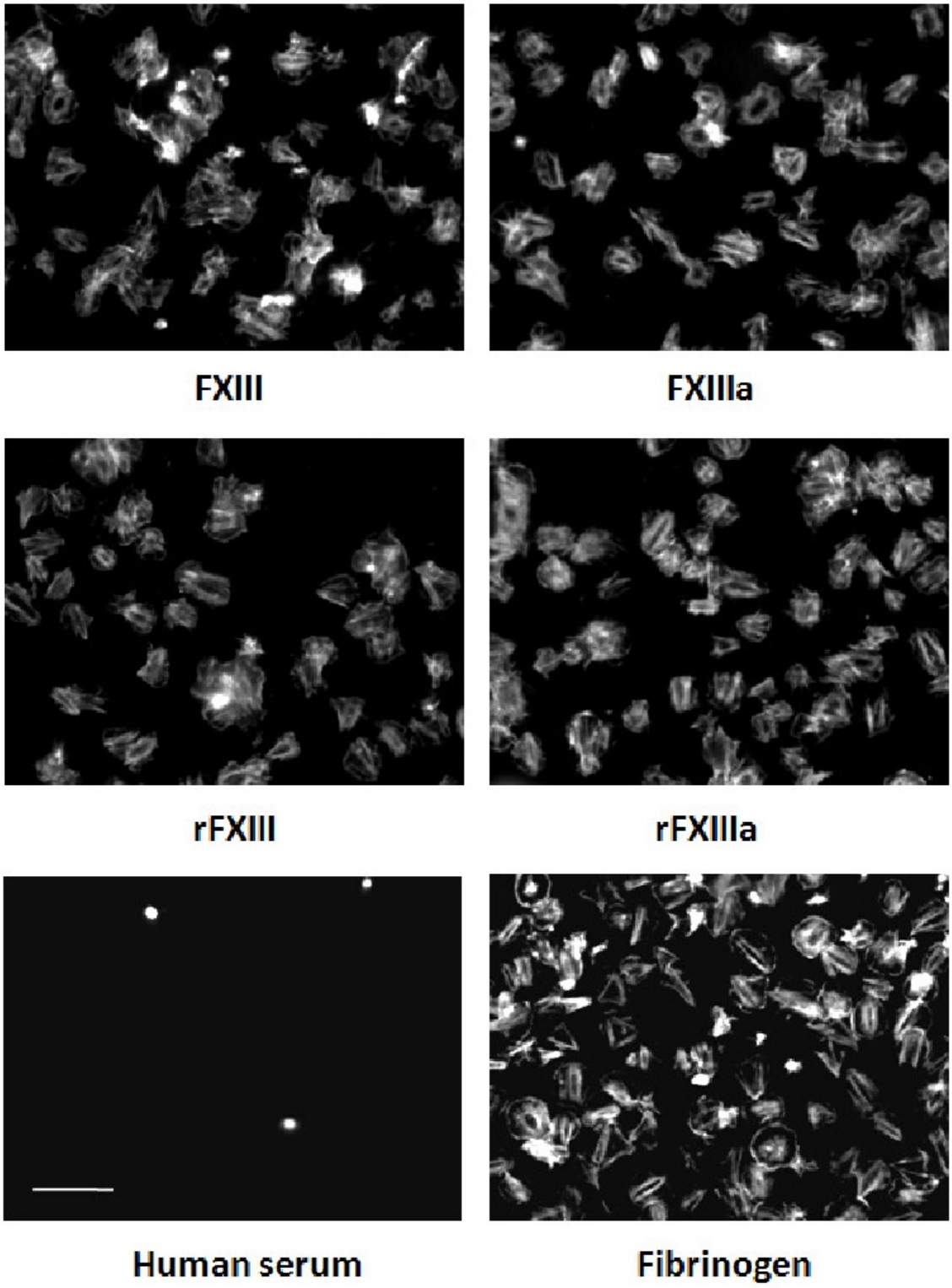
Microscope slides were coated with a range of FXIII and FXIIIa concentrations (0-400µg/mL) and platelets adhered for 1 hour. Increasing the concentration of FXIII or FXIIIa resulted in proportional increases of adherent platelet numbers (Figure 3.8). Measuring the Pearson product moment correlation coefficient (r) for the number of adherent platelets against protein concentration gave r values of 0.96 (P<0.01) and 0.98 (P<0.01) for FXIII and FXIIIa respectively. This indicated closely linear relationships between adherent platelet numbers and FXIII or FXIIIa concentration up to the highest concentration studied. Moreover,

differences in adherent platelet numbers between FXIII and FXIIIa at each concentration were statistically insignificant, suggesting that both enzyme forms had an equal tendency to support more adherent platelets with an increase in their concentration (Figure 3.8).

3.6.4 The influence of time on FXIII/FXIIIa-mediated platelet adhesion

To study the effect of time on platelet adhesion, slides were coated with FXIII and FXIIIa (both 200µg/mL) and platelets adhered for up to 90min. Increasing the incubation time led to a proportional increase in platelet adhesion to both forms of the enzyme (Figure 3.9). The association between time and adherent platelet numbers was again investigated using the Pearson product moment correlation coefficient. Here, r values of 0.98 (P<0.01) and 0.99 (P<0.01) were obtained for FXIII and FXIIIa respectively, indicating strong linear correlation between adherent platelet numbers and time during the 90 minutes studied. No statistically significant differences between platelet numbers on FXIII and FXIIIa were observed at each time point (P>0.05), suggesting that platelet adhesion on both surfaces was similarly dependent on time. In comparison, human serum did not support significant platelet adhesion even after 90min.

A)



B)

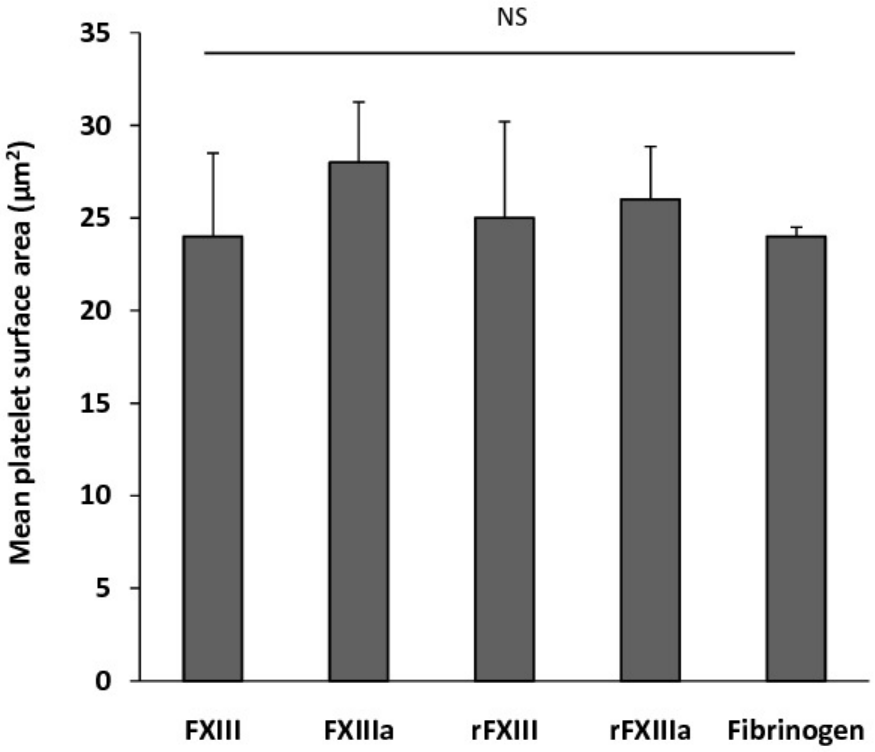


Figure 3.7: Platelets adherent on factor XIII become stimulated and spread. Wells on glass slides were coated overnight with inactive or activated factor XIII (FXIII and FXIIIa respectively, 200µg/mL), inactive or activated recombinant factor XIII (rFXIII and rFXIIIa respectively, 200µg/mL) or fibrinogen (200µg/mL). Uncoated surfaces were blocked by overlay with human serum (5%) for 30min at room temperature. Washed platelets (5×10^7 platelets/mL) were then allowed to adhere to each surface for 1hr at 37°C. Adherent platelets were stained for filamentous actin (F-actin) with TRITC-phalloidin and viewed under the ×60 oil immersion objective of an IX71 fluorescent microscope (Olympus, Japan). **(A)** Shown are representative images from 4 independent experiments with separate blood donors. **(B)** The images were used to evaluate platelet spreading using ImageJ software (NIH, USA) with data here presented as the mean platelet surface area ± SD. NS: not significant.

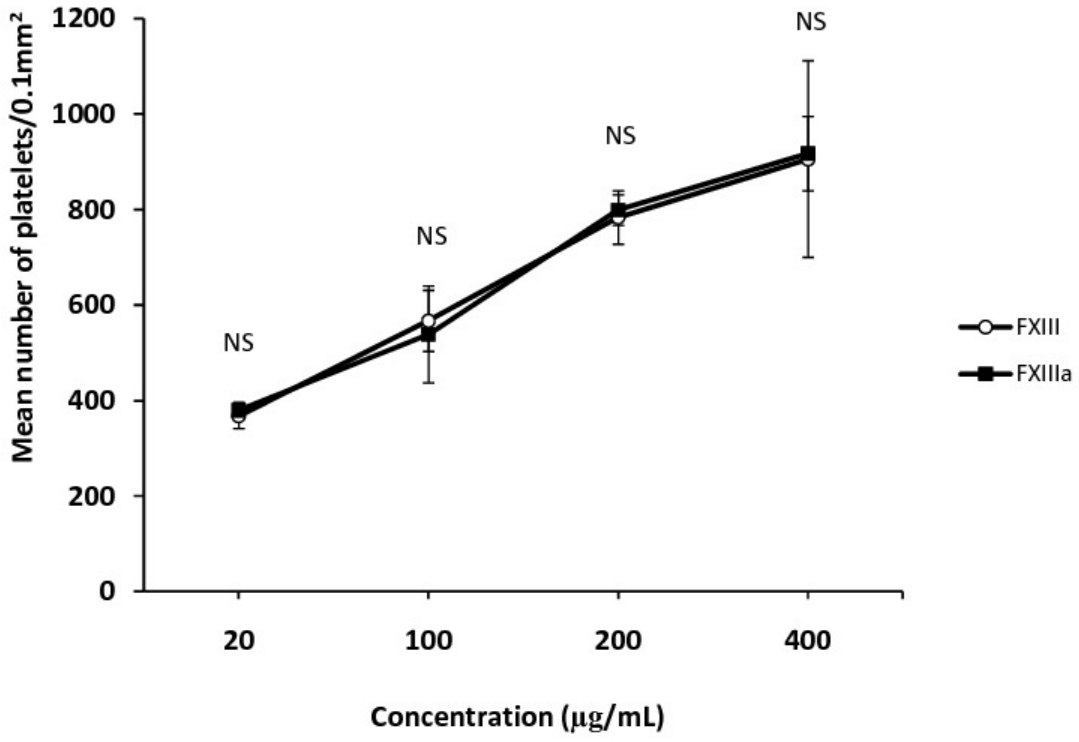


Figure 3.8: Numbers of platelets adherent to FXIII and FXIIIa increase with enzyme concentration. Glass slides were coated overnight with inactive or activated factor XIII (FXIII and FXIIIa respectively, 20-400μg/mL) and uncoated surfaces blocked by overlay with human serum (HS, 5%) for 30min at room temperature. Washed platelets (5×10^7 platelets/mL) were then adhered to either FXIII (circles) or FXIIIa (squares) for 1h at 37°C. Adherent platelets were stained with TRITC-phalloidin (10μg/mL) for 1h at room temperature then observed under the ×60 objective of an IX71 fluorescence microscope (Olympus, Japan). Platelets from random visual fields with a total area of $0.1mm^2$ were counted. Data are from 3 independent experiments with separate blood donors and are presented as mean ± SD. NS: not significant (for FXIII vs FXIIIa).

3.7 The role of enzymatic activity in platelet adhesion to FXIII

3.7.1 The influence of transglutaminase activity on platelet adhesion

The similar levels of platelet adhesion observed on FXIII and FXIIIa-coated surfaces (Figures 3.5 and 3.6) suggested that FXIII transglutaminase activity was not involved in these events. In order to confirm this, rFXIIIa was treated with iodoacetamide, an alkylating agent which irreversibly blocks its catalytic site (Curtis et al., 1974, Prasa and Sturzebecher, 2002). Consistent with a previous report (Dardik et al., 2003), incubation of rFXIIIa (200µg/mL) with iodoacetamide (3mM) inhibited transglutaminase activity of FXIII, as evidenced by a reduction in absorption values with the transglutaminase activity assay (section 2.3.2) from 1.265 to 0.110 with iodoacetamide (P<0.05) (Figure 3.10). In fact, absorption values were similar to those of rFXIII and catalase (0.103 and 0.101 P>0.05, ANOVA), indicating that iodoacetamide completely blocked FXIIIa transglutaminase activity under these conditions (Figure 3.10). Iodoacetamide-treated rFXIIIa (200µg/mL) was then immobilised on glass slides and its ability to support platelet adhesion assessed. In the control, rFXIIIa supported the adhesion of 713±82 platelets/0.1mm^2 which was maintained at 589±103 platelets/0.1mm^2 when it had been treated with iodoacetamide (P>0.05 vs rFXIIIa control) (Figure 3.11).

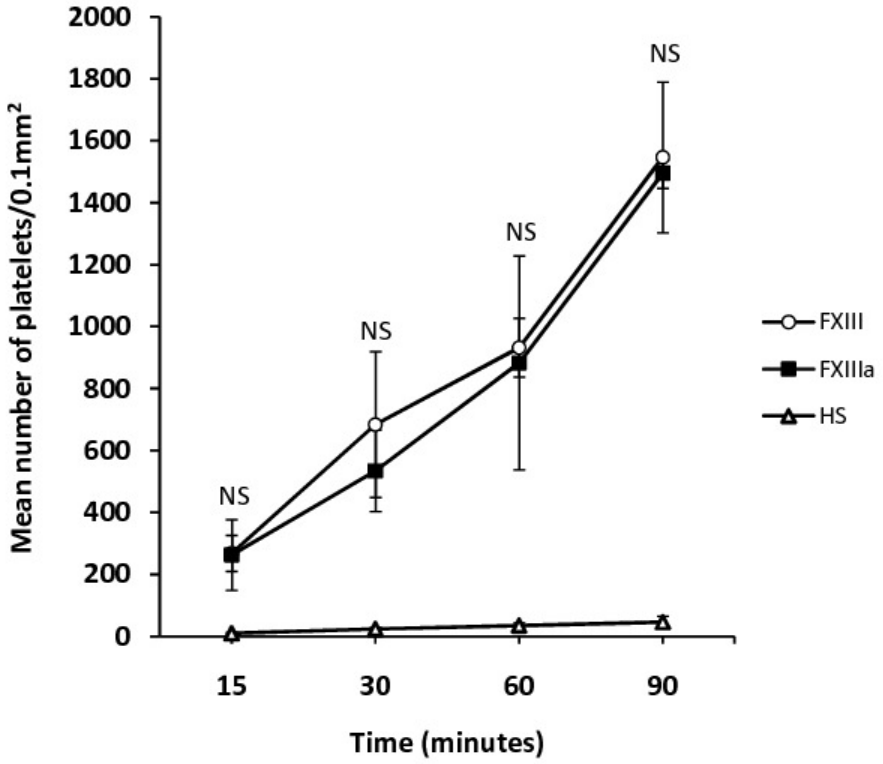


Figure 3.9: Numbers of platelets adherent to FXIII and FXIIIa increase with time. Glass slides were coated overnight with inactive or activated factor XIII (FXIII and FXIIIa respectively, 200µg/mL) and uncoated surfaces were blocked by overlay with human serum (HS, 5%) for 30min at room temperature. Washed platelets (5×10^7 platelets/mL) were then adhered to FXIII (circles), FXIIIa (squares) or HS (triangles) for different times (15-90 minutes) at 37°C. Adherent platelets were stained with TRITC-phalloidin (10µg/mL) for 1h at room temperature then observed under the ×60 objective of an IX71 fluorescence microscope (Olympus, Japan). Platelets from random visual fields were counted. Data are from 3 independent experiments with separate blood donors and are presented as mean ± SD. NS: not significant (for FXIII vs FXIIIa).

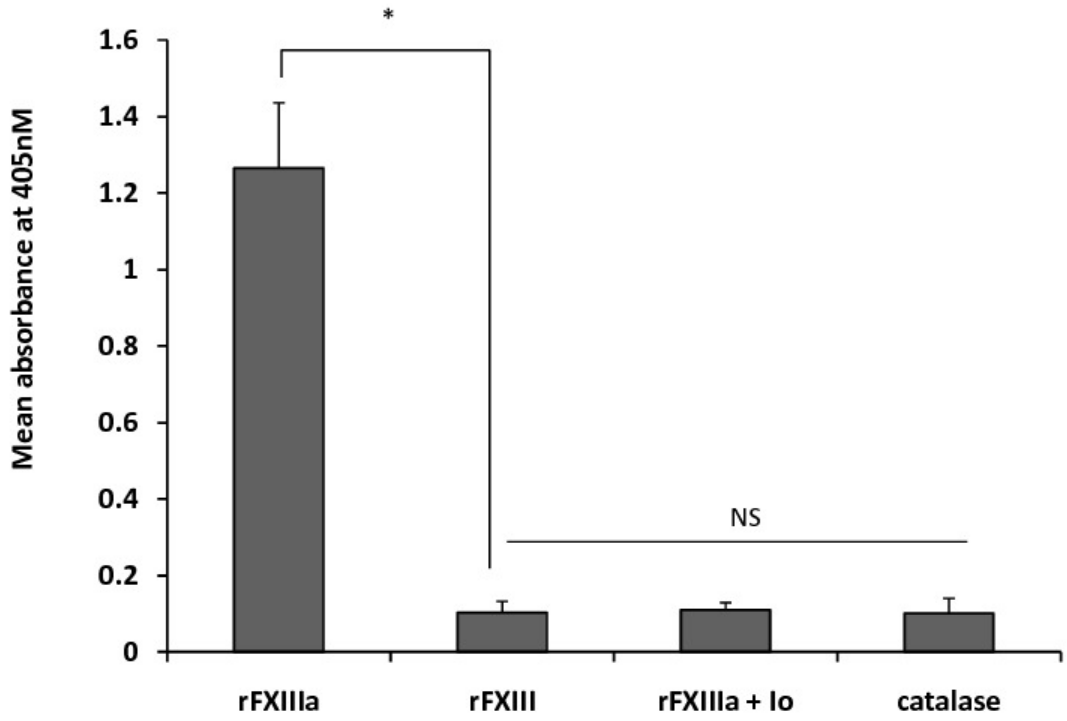


Figure 3.10: Iodoacetamide ablates factor XIII transglutaminase activity. Activated recombinant factor XIII-A (rFXIIIa, 200µg/mL) was treated with iodoacetamide (Io, 3mM) for 30min at 20°C to inhibit transglutaminase activity (Dardik et al., 2003). Transglutaminase activity was then measured by amine incorporation into fibrinogen as described by Song et al., (1994). For comparison, the transglutaminase activities of inactive recombinant factor XIII-A (rFXIII, 200µg/mL) and catalase (200µg/mL) were also measured. Data shown are mean ± SD of 3 independent experiments. *: $P<0.05$, NS: not significant.

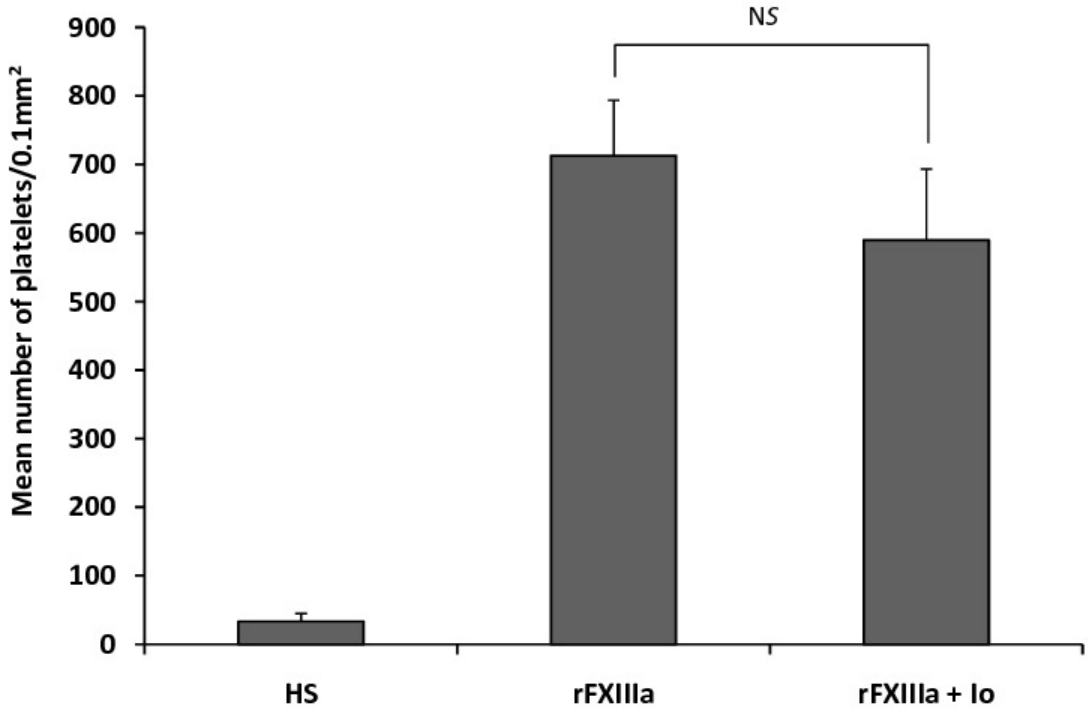


Figure 3.11: FXIIIa-mediated platelet adhesion does not depend on its transglutaminase activity. Wells on glass slides were coated overnight with activated recombinant factor XIII (rFXIIIa, 200µg/mL) and uncoated surfaces were blocked by overlay with human serum (HS, 5%) for 30min at room temperature. In some cases, rFXIIIa was incubated with iodoacetamide (3mM) at 20°C for 30min to inhibit transglutaminase activity (Dardik et al., 2003) prior to immobilisation on the slides. Washed platelets (5×10^7 platelets/mL) were then allowed to adhere on coated slides for 1h at 37°C. Adherent platelets were stained with TRITC-phalloidin (10µg/mL) for 1h at room temperature then observed under the ×60 objective of an IX71 fluorescence microscope (Olympus, Japan). Adherent platelets from random visual fields were counted. Data are from 3 independent experiments with separate blood donors and are presented as mean ± SD. NS: not significant.

3.7.2 The influence of protein disulphide isomerase activity on platelet adhesion

It had recently been reported that FXIII possesses protein disulfide-isomerase (PDI) activity which is independent of its function as a transglutaminase (Lahav et al., 2009). Thus, the potential involvement of PDI activity in platelet binding to FXIII was investigated. rFXIIIa was incubated with bacitracin (1mM), an established inhibitor of PDI activity (Mandel et al., 1993), for 1 hour at 37°C (Lahav et al., 2009), prior to measuring platelet adhesion. Incubation of FXIIIa with bacitracin did not affect platelet binding to rFXIIIa (713±82 vs 755±115 platelets/0.1mm^2 for the absence and presence of bacitracin respectively) (Figure 3.12). Together with data obtained with transglutaminase inhibitors, these results suggest that platelet binding to FXIII occurs independently of any recognised inherent enzymatic activity.

3.8 Dependence of platelet adhesion on the structure of FXIII-A

Data obtained with iodoacetamide and bacitracin suggested that platelets may bind to FXIII-A on a region(s) distinct from a catalytic site. To further investigate the availability of its binding site(s) to platelets, rFXIII and rFXIIIa were heat-denatured to distort their protein conformations. Heat-treatment prior to immobilisation ablated their ability to support platelet adhesion, diminishing it from 713±82 and 648±92 platelets/0.1mm^2 to 58±15 and 64±13 platelets/0.1mm^2 respectively (P<0.01) (Figure 3.13). This suggested a dependence of adhesion events on specific higher level FXIII protein structure.

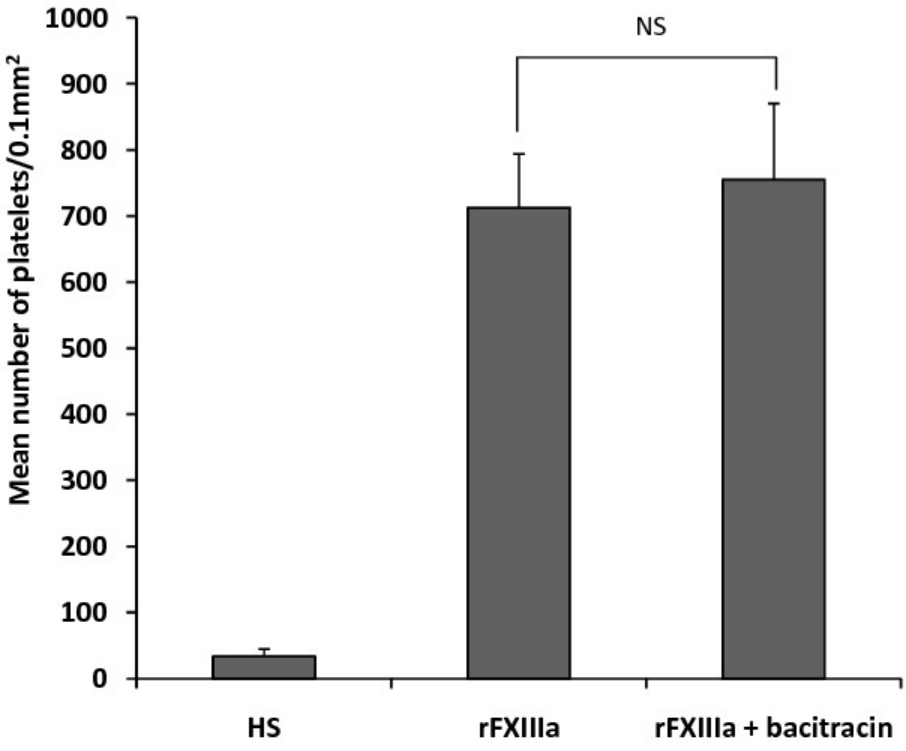


Figure 3.12: FXIII-mediated platelet adhesion does not depend on its Protein Disulphide Isomerase (PDI) activity. Wells on glass slides were coated overnight with activated recombinant factor XIII (rFXIIIa, 200µg/mL) and uncoated surfaces were blocked by overlay with human serum (HS, 5%) for 30min at room temperature. In some cases, rFXIIIa was incubated with bacitracin (1mM) at 37°C for 1h to inhibit PDI activity (Lahav et al., 2009) prior to immobilisation on the slides. Washed platelets (5×10^7 platelets/mL) were then allowed to adhere on coated slides for 1h at 37°C. Adherent platelets were stained with TRITC-phalloidin (10µg/mL) for 1h at room temperature then observed under the ×60 objective of an IX71 fluorescence microscope (Olympus, Japan). Adherent platelets from random visual fields with a total area of $0.1mm^2$ were counted. Data are from 3 independent experiments with separate blood donors and are presented as mean ± SD. NS: not significant.

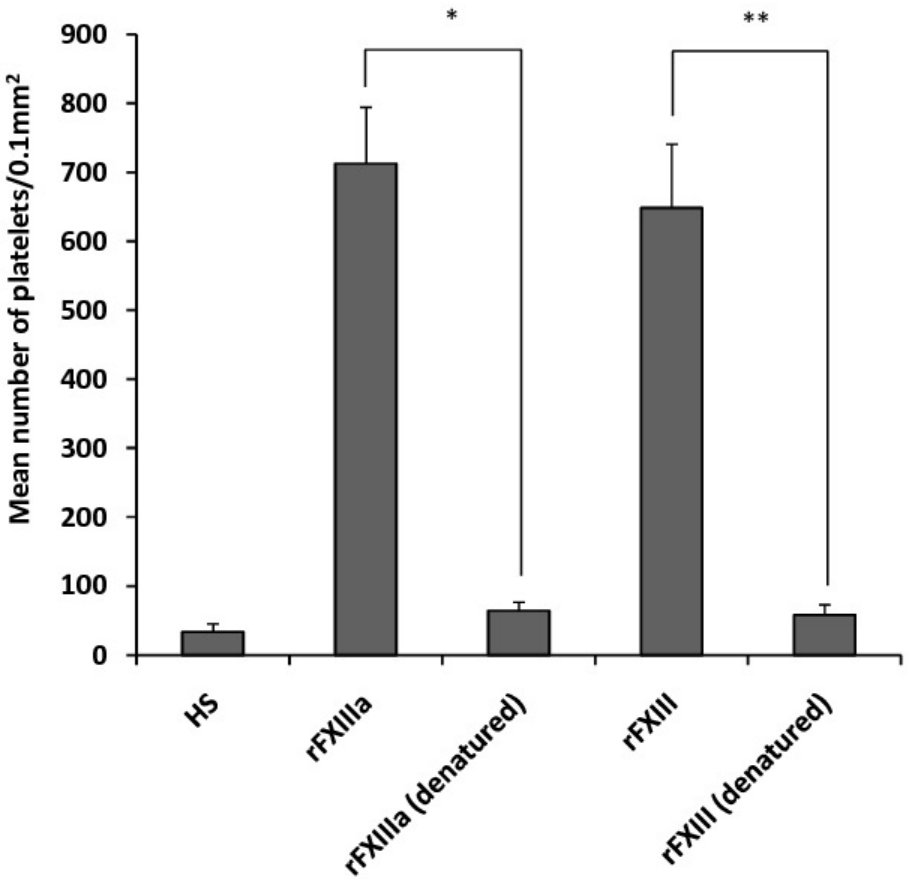


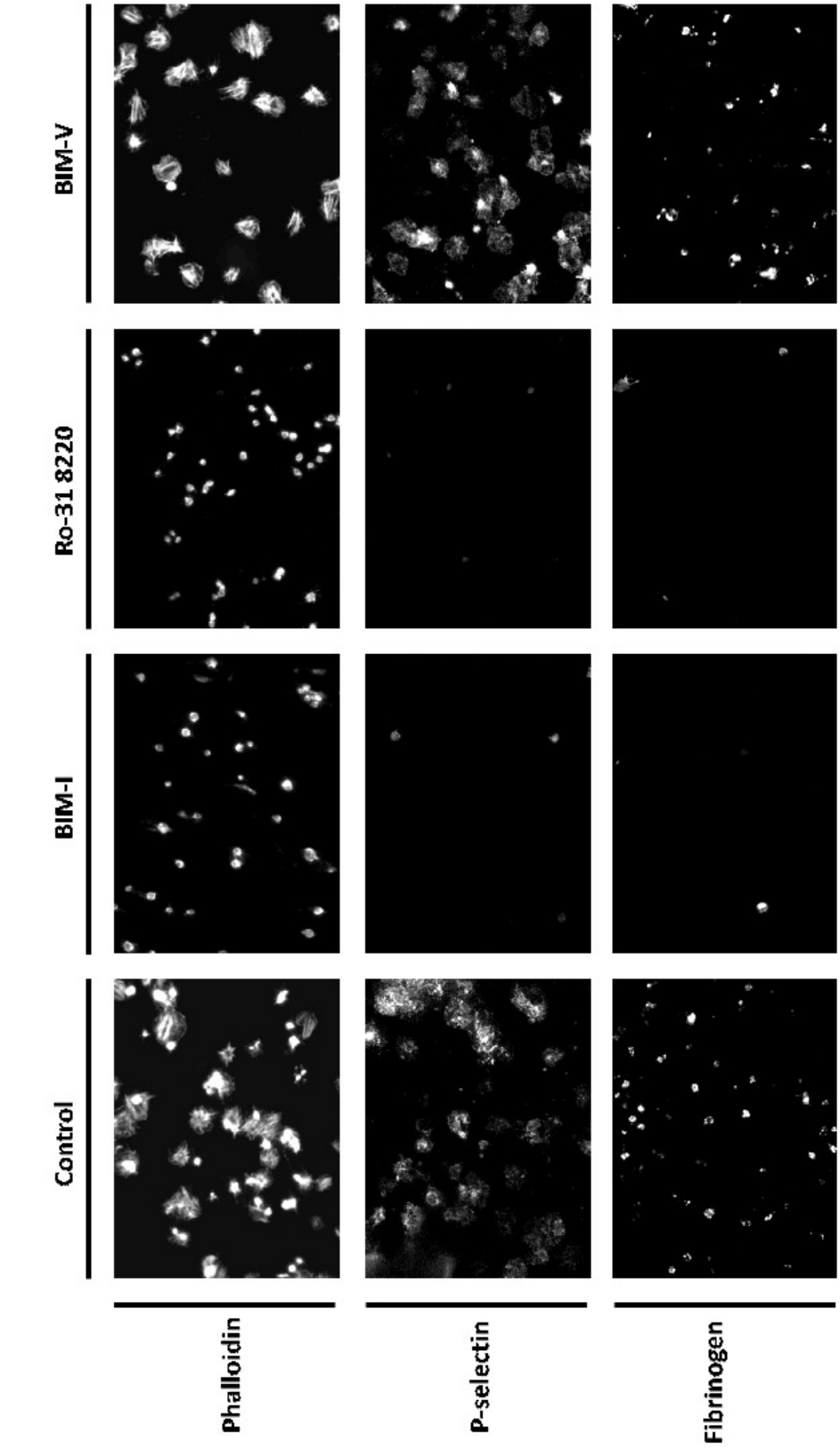
Figure 3.13: Correct factor XIII protein conformation is required for platelet adhesion. Wells on glass slides were coated overnight with inactive and activated recombinant factor XIII (rFXIII and rFXIIIa respectively, both 200µg/mL) and uncoated surfaces were blocked by overlay with human serum (HS, 5%) for 30min at room temperature. In some cases, rFXIII and rFXIIIa were denatured by incubation at 60°C for 1h prior to immobilisation on the slides. Washed platelets (5×10^7 platelets/mL) were then allowed to adhere on coated slides for 1h at 37°C. Adherent platelets were stained with TRITC-phalloidin (10µg/mL) for 1h at room temperature then observed under the ×60 objective of an IX71 fluorescence microscope (Olympus, Japan). Adherent platelets from random visual fields were counted. Data are from 3 independent experiments with separate blood donors and are presented as mean ± SD. *: $P<0.05$, **: $P<0.01$.

3.9 Platelet adhesion to FXIII occurs independently of proteins secreted from α-granules

Platelet α-granules contain vWF, fibrinogen, thrombospondin and fibronectin which are ligands for platelet adhesion as well as FXIII substrates (Muszbek et al., 1999). While the absence of contaminating proteins from Fibrogammin P and rFXIII-A had been established (section 3.2), it remained feasible for platelet binding to be indirectly mediated by secreted α-granule proteins which interacted with the immobilised FXIIIa surface.

To determine whether FXIIIa-adherent platelets indeed secreted their α-granule contents, adherent platelets were probed with FITC-conjugated antibodies against surface P-selectin and fibrinogen, two markers for α-granule secretion (Holmsen and Weiss, 1979, Rendu and Brohard-Bohn, 2001). Adherent platelets secreted their α-granule contents as indicated by increased expression of both P-selectin and fibrinogen on their surfaces (Figure 3.14A). To assess whether secreted α-granule proteins contributed to platelet adhesion, platelets were treated with PKC antagonists that inhibit granular secretion (Konopatskaya et al., 2009, Harper and Poole, 2010) prior to adhesion. The PKC antagonists Ro-31-8220 and BIM I (both 10μM) prevented secretion as evidenced by a lack of surface-expressed fibrinogen and P-selectin. However, BIM V (10μM), an inactive analogue was without effect (Figure 3.14A). Importantly, the extent of platelet adhesion was unaffected by inhibiting secretion since adherent platelet numbers in the control (850±246 platelets/0.1mm^2) were maintained under treatment with Ro-31-8220, BIM I and BIM V (705±93, 781±57 and 695±57 platelets/0.1mm^2 respectively; P>0.05, ANOVA) (Figure 3.14B).

Inhibition of granular secretion by Ro-31-8220 and BIM I was confirmed by lumi-aggregometry (section 2.7.2). Here, platelet dense granule secretion induced by PMA (100nM), a specific PKC agonist, was abolished by BIM I and Ro-31-8220, but unaffected by BIM V (Figure 3.15). These data together with those showing platelet adhesion to plasma protein-free recombinant FXIII-A (Figure 3.6), strongly suggest that FXIII-A supports platelet adhesion independently of other proteins.


Control
BIM-I
Ro-31 8220
BIM-V
Phalloidin
P-selectin
Fibrinogen

A)

B)

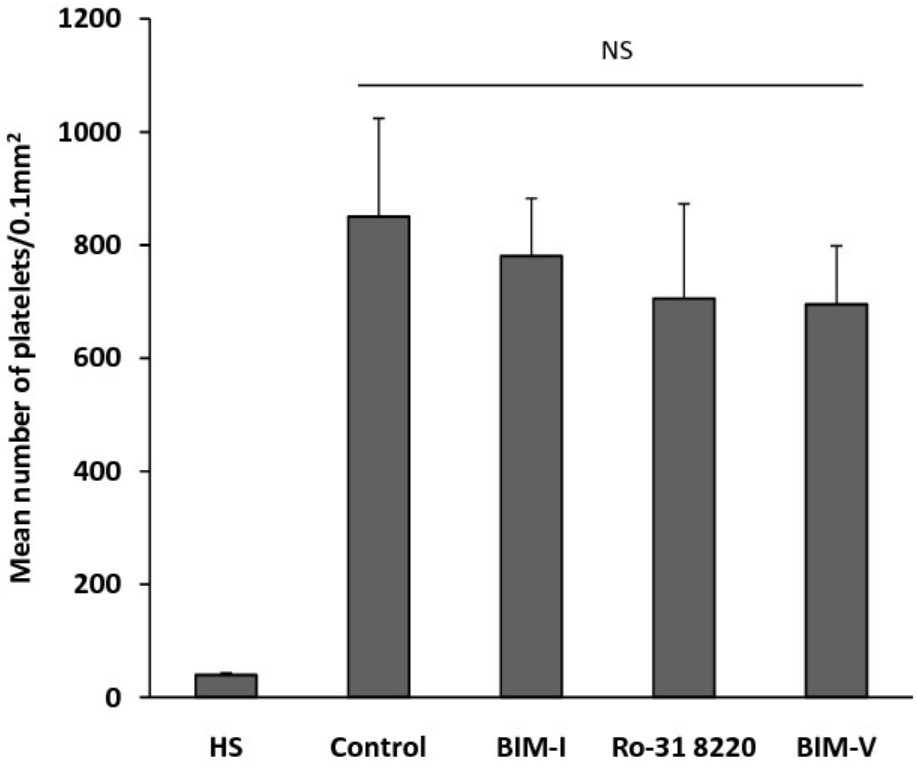


Figure 3.14: Platelet adhesion to FXIII is independent of secreted granular proteins. Wells on glass slides were coated overnight with FXIIIa (200µg/mL) and uncoated surfaces were blocked by overlay with human serum (HS, 5%) for 30min at room temperature. Washed platelets (5×10^7 platelets/mL) were incubated with the protein kinase C antagonists Ro-31-8220 and BIM I (both 10µM), their inactive analogue BIM V (10µM) or 0.01% DMSO (control) for 20min at 37°C. Treated platelets were allowed to adhere to the coated slides for 1hr at 37°C. In some cases, adherent platelets were lysed to permeabilise them, followed by overlay with TRITC-conjugated phalloidin (10µg/mL) for 1hr at room temperature. In all other instances, lysis was omitted and surface expressed fibrinogen and P-selectin were probed using specific FITC-conjugated antibodies for 1hr at room temperature. Platelets were then viewed under a fluorescence microscope. **(A)** Shown are representative images from 3 independent experiments. **(B)** Platelets adherent to FXIIIa following each treatment were counted and are presented as mean ± SD. Data are from 3 independent experiments with separate blood donors. NS: not significant.

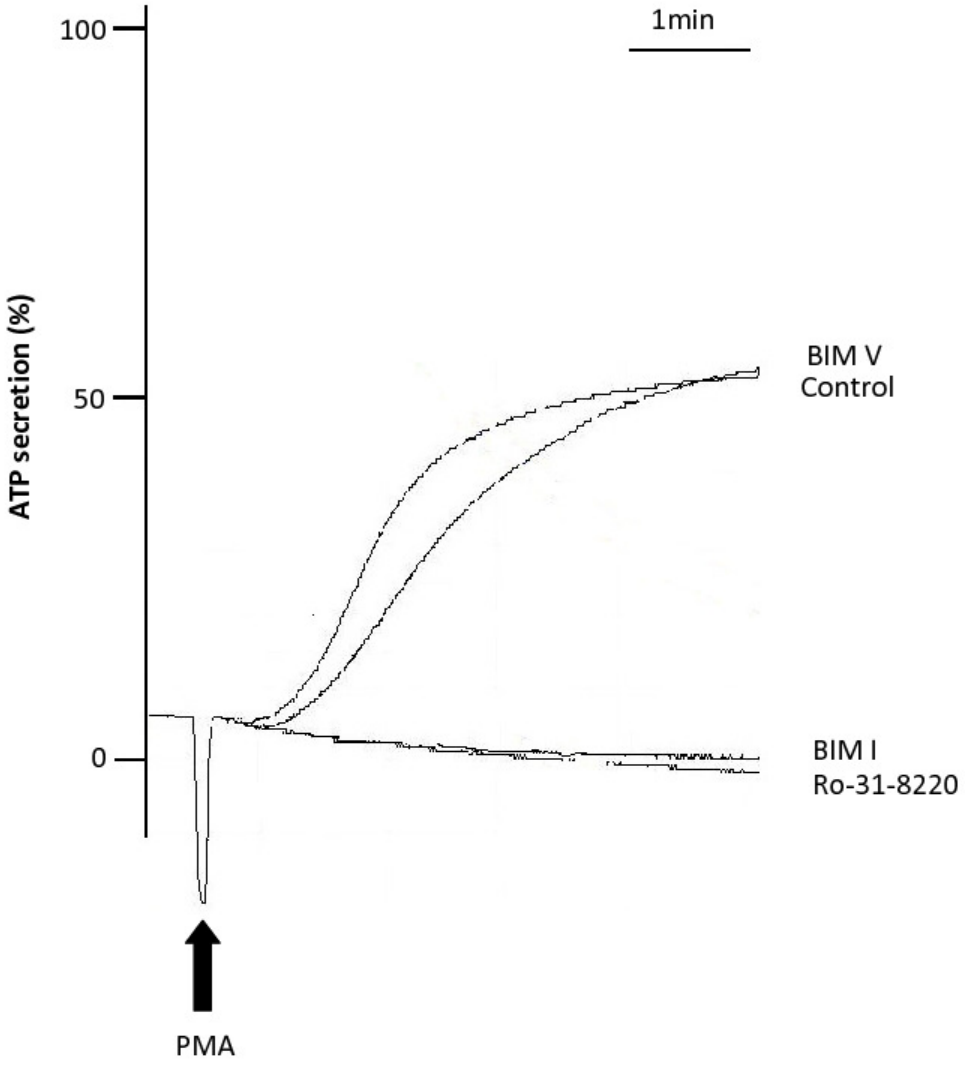


Figure 3.15: Protein kinase C (PKC) inhibitors block platelet granular secretion. Washed platelets (3×10^8 platelets/mL) were incubated with the PKC antagonists Ro-31-8220 and BIM I (both 10µM) or their inactive analogue BIM V (10µM) for 20min at 37°C. Secretion of adenosine triphosphate (ATP) from platelet dense granules was stimulated with phorbol myristate acetate (PMA, 100nM) under constant stirring conditions (1000rpm) and was measured over 4min using a Chrono-log dual light-transmission and luminescence aggregometer. Luciferin/luciferase (2nM) and ATP (1nM) were used as standards for machine calibration. Curves were recorded by Aggro/Link software from Chrono-log.

3.10 Discussion

Interactions between platelets and FXIII were first studied about 25 years ago (Greenberg and Shuman, 1984). Since then, subsequent studies have focused almost exclusively on characterising platelet-FXIII interactions in suspension, yielding conflicting results. While some investigators argued that the ability to bind platelets is restricted to FXIIIa (Greenberg and Shuman, 1984, Cox and Devine, 1994), an unconfirmed report suggested that suspended FXIII may also bind platelets (Dardik et al., 2002b). In the same study, immobilised FXIII and FXIIIa were also shown to support platelet adhesion. However, possible background activation of FXIII caused by experimental procedures was not excluded. For instance, it is known that FXIII can become activated by conformational change even in the absence of thrombin cleavage (Polgar et al., 1990, Muszbek et al., 1993). Since proteins are likely to have altered conformations when immobilised on electrostatic surfaces such as glass and plastic (Baugh and Vogel, 2004), FXIII could have become activated upon immobilisation resulting in only circumstantial evidence for the redundancy of transglutaminase activity in platelet binding.

In the present project, particular focus was made on studying platelet associations with immobilised FXIII due to its ability to localise at sites of vascular injury either by interacting with the ECM (Mosher, 1984, Kikuchi et al., 1986, Sane et al., 1988) or through its associations with fibrinogen chains entangled within blood clots (Greenberg and Shuman, 1982, Siebenlist et al., 1996). Using established methodology to investigate platelet adhesion

and spreading on protein coated surfaces, the adhesive capacities of FXIII and FXIIIa were explored.

Since Fibrogammin P contained FXIII sourced from plasma and FXIII circulates bound to fibrinogen (Siebenlist et al., 1996), a major platelet ligand, care was taken to ensure that potential platelet-adhesive contaminants were absent from Fibrogammin P prior to investigating its ability to support platelet adhesion. Using gel electrophoresis and western blotting, FXIII and albumin were the only two proteins detectable in Fibrogammin P (Figures 3.1A and 3.2). Given that platelets do not adhere to the latter, it was not expected to affect the results of adhesion experiments. In fact, albumin is the major protein component of human serum which was used as a blocking agent to prevent platelet adhesion to glass (section 2.5.2.1). Crucially, fibrinogen was undetectable from Fibrogammin P using both gel-staining and immunoblotting techniques (Figure 3.1). After this rigorous testing, observations of platelet adhesion on immobilised Fibrogammin P were more confidently believed to be mediated by FXIII. This was confirmed by reproducible platelet adhesion on immobilised recombinant FXIII-A which was not exposed to plasma proteins throughout its synthesis (Figure 3.6). A remaining possibility was the secretion of adhesive proteins from platelet α-granules and their subsequent localisation on immobilised FXIII. However, effective inhibition of platelet secretion failed to affect platelet binding (Figure 3.14). Taken together, these data strongly suggest that platelet binding to FXIII can occur independently of other plasma proteins. Moreover, such adhesion is stimulatory, resulting in spreading (Figure 3.7) and degranulation responses (Figure 3.14A).

Platelet stimulation appears to be a unique property of immobilised FXIII since it failed to stimulate platelet shape change or aggregation when added to platelets in suspension (Figure 3.4A). This may ensure that FXIII-mediated platelet recruitment and activation are restricted to sites of vascular injury where FXIII may become localised (Kikuchi et al., 1986).

Consistent with the report from Dardik et al., (2002b) adhesion experiments revealed that under static conditions, both FXIII and FXIIIa supported equal platelet adhesion. This was confirmed using two different techniques; a microplate-based method from Bellavite et al., (1994) (Figure 3.5) and a fluorescence microscopy-based adhesion assay (Figure 3.6). The immediate implication from these data was the redundancy of transglutaminase activity in FXIII-mediated platelet binding. This was confirmed by unchanged levels of platelet adhesion to rFXIIIa following direct inhibition of its transglutaminase activity with iodoacetamide (Figure 3.11). These data therefore suggest that different mechanisms control platelet and fibroblast binding to immobilised FXIII since a strict dependence on transglutaminase activity was demonstrated for the latter (Ueki et al., 1996). However, consistent with the present platelet data, endothelial cells were shown to adhere to immobilised FXIIIa independently of its transglutaminase activity (Dallabrida et al., 2000).

If the platelet-binding site(s) on FXIIIa is indeed separate from its catalytic site, it is tempting to speculate that platelet-bound FXIIIa remains free to catalyse cross-linkages among its substrates on the platelet surface. In fact, FXIIIa was attributed a critical role in the development of procoagulant "coated" platelets by cross-linking secreted α-granule proteins such as vWF, thrombospondin, factor V, vitronectin and fibronectin to serotonin on

platelet surfaces (Dale et al., 2002). Although the PDI activity of FXIII is also not required for platelet adhesion (Figure 3.12), it may have a potential haemostatic role since PDI-mediated alteration of disulphide bonds in $\alpha_{IIb}\beta_3$ integrins can induce platelet aggregation (O'Neill et al., 2000). However, the physiological consequences of these events and their feasibility *in vivo* are yet to be fully determined.

Platelet binding to FXIII correlated positively with enzyme concentration (Figure 3.8) and time (Figure 3.9). Similar dependence on time and concentration was observed for platelet adhesion to both FXIII and FXIIIa. This suggests that the platelet-binding site(s) on both enzyme forms are equally accessible to platelets. Their availability to platelets is dependent on correct protein conformation since platelets failed to bind to denatured FXIII (Figure 3.13). The importance of these observations derives from recent reports that elevated FXIII plasma levels are associated with predisposition to chronic cardiovascular diseases such as MI (Bereczky et al., 2007) and PVD (Shemirani et al., 2008, Kloczko et al., 1988). Considering that more platelets are recruited to surfaces coated with increasing amounts of FXIII (Figure 3.8), it is likely that platelet-FXIII interactions may play a role in events leading to these pathologies. Such a paradigm is supported by evidence that when *in vivo* FXIII concentrations are raised, platelets more readily bind to activated endothelium typically found in atherosclerotic regions of the vasculature (Dardik et al., 2002b). Furthermore, patients with PVD have platelets which circulate bound to more FXIII than those of matched controls without the disease (Devine et al., 1993).

In conclusion, the data presented in this chapter have broadly outlined the basic observations supporting a hypothesised interaction between platelets and immobilised FXIII which may have a pathophysiological preponderance. Successive chapters will build upon these findings by describing them in finer molecular detail and assessing the practicality of their occurrence.

CHAPTER 4 - INTEGRIN-MEDIATED PLATELET ADHESION TO FXIII ENHANCES THROMBUS FORMATION BY EXTRACELLULAR MATRIX PROTEINS UNDER FLOW.

4.1 Introduction

The integrin $\alpha_{IIb}\beta_3$ is believed to facilitate platelet-FXIII binding (Cox and Devine, 1994). However, platelets from patients with GT retained an ability to bind FXIII despite lacking functional $\alpha_{IIb}\beta_3$ (Greenberg and Shuman, 1984), suggesting the involvement of additional platelet receptor(s).

Physiologically, platelets are exposed to an array of blood flow conditions. In veins where blood velocity is low, typical wall shear rates are less than 500 s^{-1} whereas in small arterioles, shear rates can reach 5000 s^{-1} (Tangelder et al., 1988). Moreover, under pathological conditions such as those found in stenosed arteries, shear rates as high as 40 000 s^{-1} have been reported (Bluestein et al., 1997). During thrombus formation, primary platelet adhesion occurs on exposed subendothelial ECM proteins, primarily collagen, resulting in the deposition of a platelet monolayer (Farndale et al., 2004). Both primary platelet adhesion and aggregation are influenced by prevailing haemodynamic conditions (Ruggeri and Mendolicchio, 2007). Platelets are able to form adhesive contacts under all shear conditions. However, at shear rates higher than 800s^{-1}, platelet interaction with vWF becomes critical for both primary adhesion and aggregation which drives increase in thrombus volume (Weiss et

al., 1978b). It is noteworthy that this important role for vWF in platelet thrombus formation is not immediately apparent when measuring platelet function using optical aggregometry. This is because platelets are able to aggregate by binding fibrinogen in the absence of vWF under the low shear rates generated in this technique (Jackson, 2007).

Previous studies have demonstrated the ability of platelets to bind FXIII in suspension (Greenberg and Shuman, 1984, Cox and Devine, 1994). Dardik et al., (2002b) also reported that immobilised FXIII could mediate platelet accrual on activated endothelium. However, the occurrence and stability of platelet-FXIII associations with the immobilised enzyme under physiological shear have largely remained uncharacterised and their relevance with respect to platelet adhesion to the ECM has not been investigated. Due to normal platelet responses under optical aggregometry in FXIII-deficient platelets (Ozsoylu and Hicsonmez, 1976), FXIII was considered a minor contributor to haemostatic platelet function. However, in light of observations which revealed the importance of vWF to platelet thrombus formation (Savage et al., 1998), it is important that platelet-FXIII interactions are also examined under physiological flow conditions

The ability of FXIII to support platelet adhesion under static conditions was described in Chapter 3. Analyses of adhesion events under these conditions were useful for characterising responses stimulated in a primary adherent monolayer, particularly spreading events (section 3.6). However, since blood flow exerts considerable influence on platelet function, it was important that observations made under static conditions were revaluated for stringency under physiological haemodynamic conditions.

Aims:

- To identify potential platelet receptors that mediate adhesion to immobilised FXIII.
- To characterise the functional roles of each receptor type in the adhesion process.
- To determine whether adhesive contacts between platelets and FXIII occur in flowing blood.
- To examine how such contacts are influenced by differential shear rates that accompany the range of blood velocities found physiologically in different vascular regions.
- To test whether FXIII influences platelet thrombus formation on established platelet adhesive ligands under flow conditions.

4.2 The role of integrins in platelet adhesion to immobilised FXIIIa

4.2.1 Platelet adhesion to FXIIIa is integrin-mediated

Since previous reports had alluded to integrin involvement in platelet adhesion to suspended FXIIIa (Cox and Devine, 1994, Nagy et al., 2009), experiments were performed to examine whether platelet adhesion to immobilised FXIIIa was also integrin dependent. Washed platelets (5×10^7 platelets/mL) were incubated with RGDS (500μM), an integrin-blocking peptide that competitively inhibits multiple integrins (Ruoslahti, 1996), prior to adhesion to FXIIIa. RGDS diminished platelet adhesion from 921±128 to 102±45 platelets/0.1mm^2 (P<0.01) (Figure 4.1). Importantly, platelet treatment with RGDS reduced platelet adhesion to levels similar to those occurring residually on human serum-coated surfaces (36±13 platelets/0.1mm^2, P>0.05 vs RGDS). These data showed that integrins were indispensable for adhesion to immobilised FXIIIa.

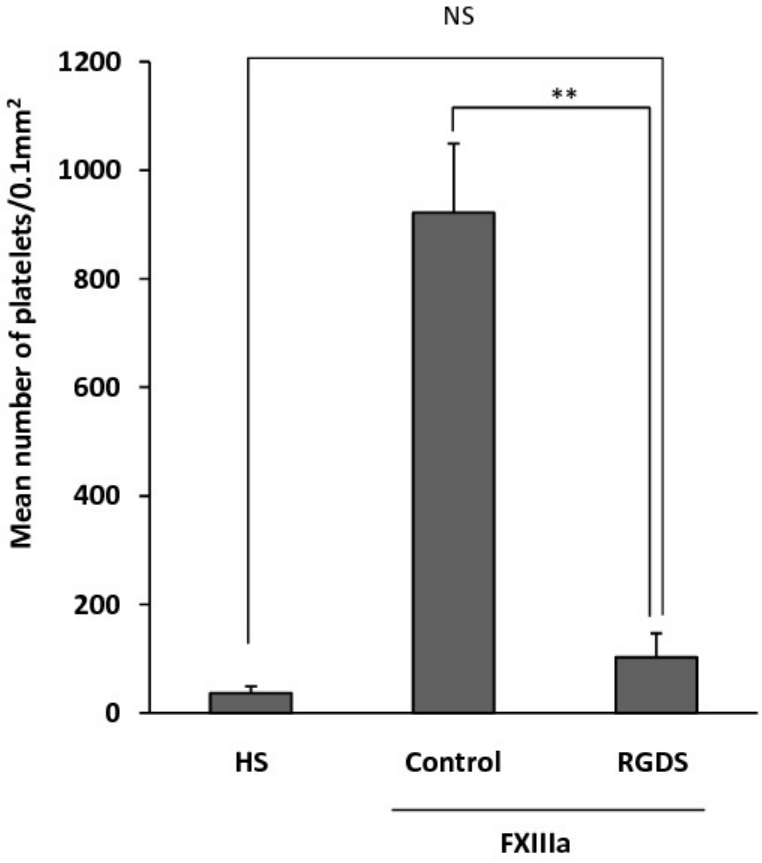


Figure 4.1: Platelet adhesion to FXIIIa is integrin dependent. Wells on glass slides were coated overnight with heat-inactivated human serum (HS, 5%) or activated FXIII (FXIIIa, 200µg/mL). Uncoated surfaces were blocked by overlay with HS (5%) for 30min at room temperature. Washed platelets (5×10^7 platelets/mL) were preincubated with RGDS (500µM) or modified Tyrode's buffer (control) for 20min at 37°C then allowed to adhere to the indicated surface for 1 hour at 37°C. Adherent platelets were stained with TRITC-phalloidin and viewed under the ×60 objective of an IX71 fluorescence microscope (Olympus, Japan). Platelets from random visual fields with a total area of $0.1mm^2$ were counted. Data shown are means ± SD and are from 4 independent experiments with separate blood donors. **: $P<0.01$, NS: not significant.

4.2.2 Platelet-FXIIIa binding is mediated by $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$

After establishing the general involvement of integrins in binding immobilised FXIIIa, the specific integrin subtypes involved were identified. Platelets were incubated with tirofiban (2μM); a specific $\alpha_{IIb}\beta_3$ antagonist prior to adhesion to FXIIIa. Tirofiban at a concentration of 2μM has been shown to completely inhibit $\alpha_{IIb}\beta_3$ integrins (Tanaka et al., 2004, Serebruany et al., 2007). Treatment with tirofiban partially reduced platelet adhesion to FXIIIa from 941±42 to 566±72 platelets/0.1mm^2 (P<0.05) (Figure 4.2), demonstrating a role for $\alpha_{IIb}\beta_3$ in this process. However, since the inhibitory effect with tirofiban was significantly less than that obtained with RGDS (103±90 platelets/0.1mm^2; P<0.05), it suggested the involvement of other integrin receptors. Moreover, inhibition of platelet adhesion to FXIIIa with tirofiban differed from that of adhesion to fibrinogen since tirofiban almost abolished platelet adhesion to the latter (1711±134 to 156±25 platelets/0.1mm^2 for the absence and presence of tirofiban respectively, P<0.01) (Figure 4.3).

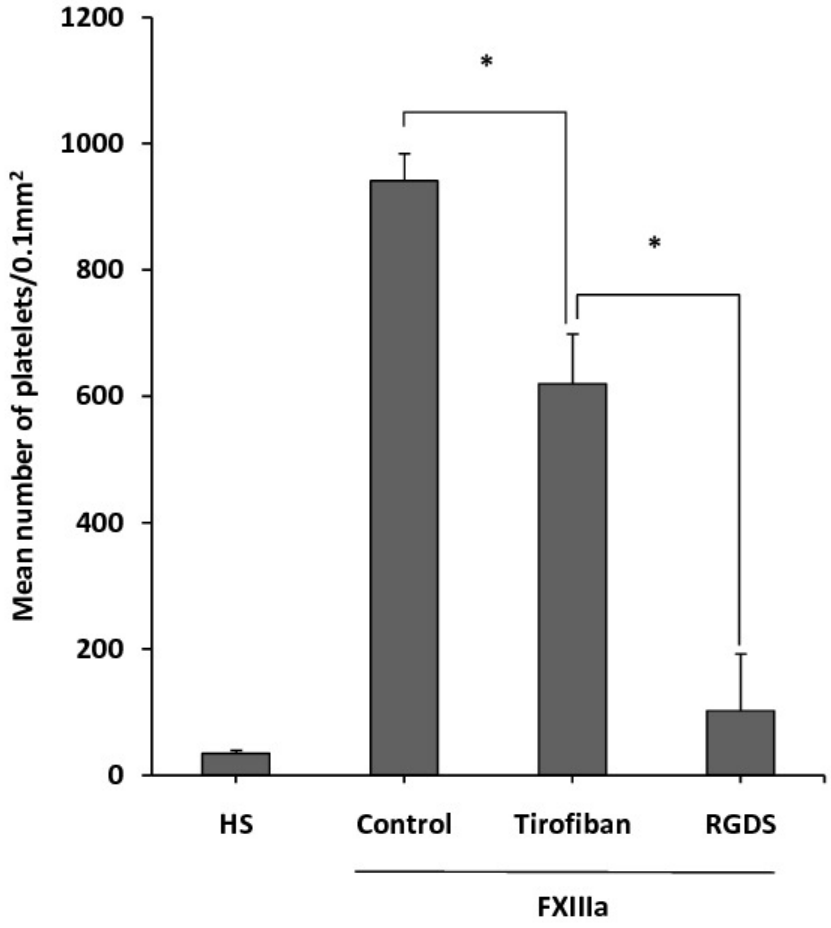


Figure 4.2: Platelet adhesion to FXIIIa is partially dependent on $\alpha_{IIb}\beta_3$ integrins. Wells on glass slides were coated overnight with heat-inactivated human serum (HS, 5%) or activated factor XIII (FXIIIa, 200µg/mL). Uncoated surfaces were blocked by overlay with HS (5%) for 30min at room temperature. Washed platelets (5×10^7 platelets/mL) were preincubated with tirofiban (2µM), RGDS (500µM) or modified Tyrode's buffer (control) for 20min at 37°C then allowed to adhere to each surface for 1h at 37°C. Adherent platelets were stained with TRITC-phalloidin and viewed under the ×60 objective of an IX71 fluorescence microscope (Olympus, Japan). Platelets from random visual fields with a total area of $0.1mm^2$ were counted. Data shown are means ± SD and are from 4 independent experiments with separate blood donors. *: $P<0.05$.

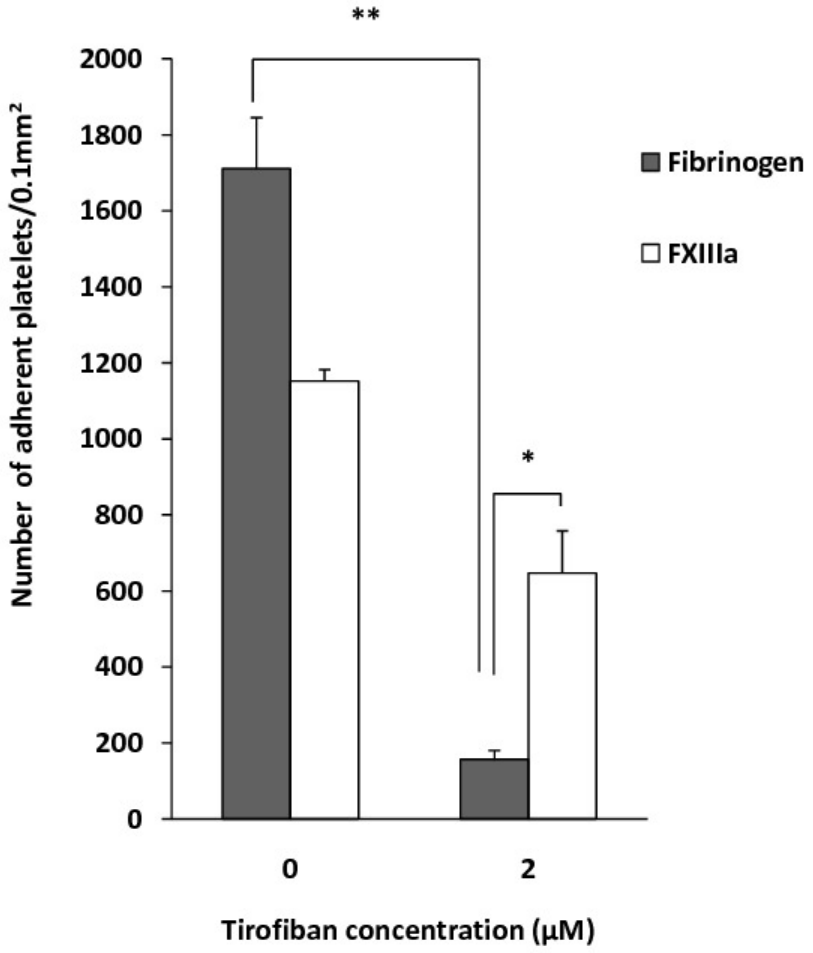


Figure 4.3: Tirofiban ablates platelet adhesion to fibrinogen, but not FXIIIa. Wells on glass slides were coated overnight with fibrinogen (200µg/mL, grey bars) or activated factor XIII (FXIIIa) (200µg/mL, white bars). Uncoated surfaces were blocked by overlay with heat-inactivated human serum (5%) for 30min at room temperature. Washed platelets (5×10^7 platelets/mL) were preincubated with tirofiban (2µM) or modified Tyrode's buffer for 20min at 37°C then allowed to adhere to each surface for 1 hour at 37°C. Adherent platelets were stained with TRITC-phalloidin and viewed under the ×60 objective of an IX71 fluorescence microscope (Olympus, Japan). Platelets from random visual fields with a total area of $0.1mm^2$ were counted. Data shown are means ± SD and are from 3 independent experiments with different blood donors. **: $P<0.01$, *: $P<0.05$.

The integrin $\alpha_v\beta_3$ mediates monocyte and endothelial cell adhesion to FXIIIa (Ueki et al., 1996, Dallabrida et al., 2000, Dardik et al., 2002b). It was likely that it also plays a similar role in platelets since they also express $\alpha_v\beta_3$ (Lam et al., 1989). Platelets were therefore incubated with LM609 (20µg/mL); a specific $\alpha_v\beta_3$-blocking antibody (Cheresh and Spiro, 1987, Brooks et al., 1994). LM609 at a concentration of 20µg/mL has previously been shown to fully inhibit $\alpha_v\beta_3$ integrins (Soldi et al., 1999, May et al., 2002). Treatment with LM609 significantly diminished platelet adhesion to FXIIIa from 941±42 to 480±91 platelets/0.1mm^2 (P<0.01) (Figure 4.4). However, similar to inhibition by tirofiban, the effect was less than that observed with RGDS (103±90 platelets/0.1mm^2, P<0.05 vs LM609). Importantly, no inhibition of platelet adhesion was observed when platelets were incubated with a control antibody that was non-reactive against $\alpha_v\beta_3$ and isotype-matched with LM609 (886±66 platelets/0.1mm^2 P>0.05 vs control). Hence the effects observed with LM609 were not due non-specific platelet-antibody interactions. LM609 did not affect platelet adhesion to fibrinogen (1769±44 vs 1960±42 for the absence and presence of LM609 respectively, P>0.05) (Figure 4.5). Together with data obtained using tirofiban (Figure 4.3), this further distinguished the roles of platelet $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins in mediating adhesion to fibrinogen and FXIIIa.

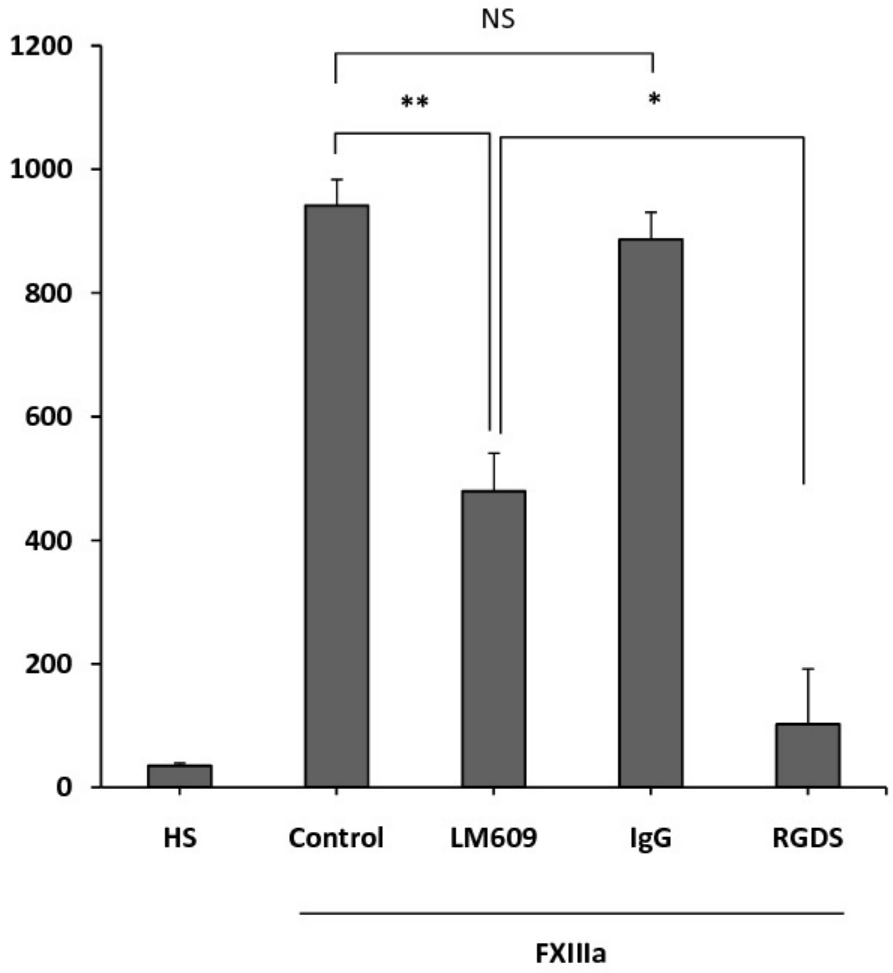


Figure 4.4: Platelet adhesion to FXIIIa is partially dependent on $\alpha_v\beta_3$ integrins. Wells on glass slides were coated overnight with heat-inactivated human serum (HS, 5%) or activated factor XIII (FXIIIa, 200µg/mL). Uncoated surfaces were blocked by overlay with HS (5%) for 30min at room temperature. Washed platelets (5×10^7 platelets/mL) were preincubated with LM609 (20µg/mL), normal mouse IgG (20µg/mL), RGDS (500µM) or modified Tyrode's buffer (control) for 20min at 37°C then allowed to adhere on the indicated surface for 1hr at 37°C. Adherent platelets were stained with TRITC-phalloidin and viewed under the ×60 objective of an IX71 fluorescence microscope (Olympus, Japan). Platelets from random visual fields with a total area of $0.1mm^2$ were counted. Data shown are means ± SD and are from 3 independent experiments with different blood donors. **: $P<0.01$, *: $P<0.05$, NS: not significant.

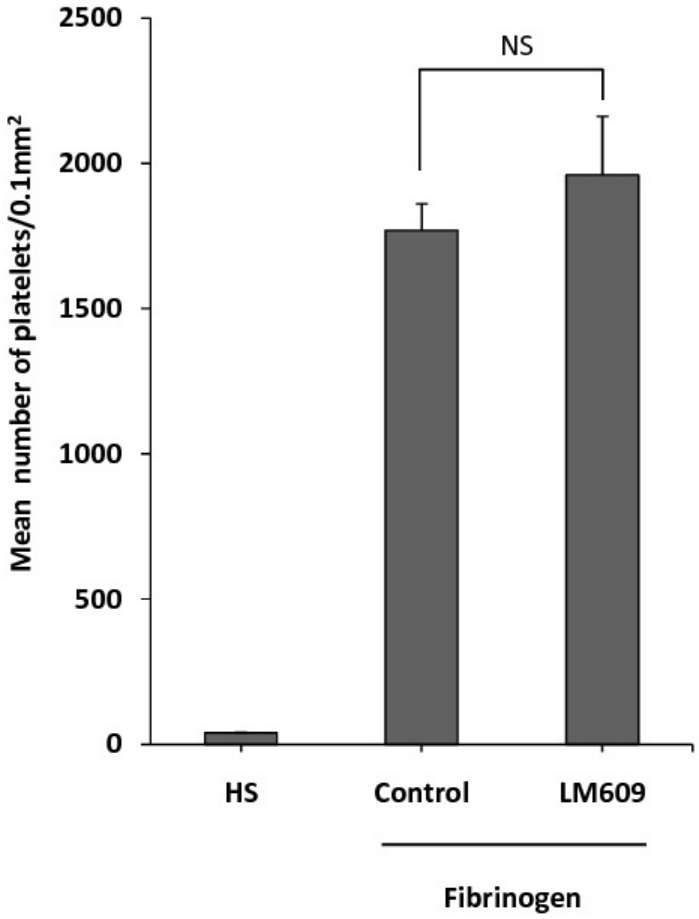


Figure 4.5: LM609 does not affect platelet adhesion to fibrinogen. Wells on glass slides were coated overnight with heat-inactivated human serum (HS, 5%) or fibrinogen (200µg/mL). Uncoated surfaces were blocked by overlay with HS (5%) for 30min at room temperature. Washed platelets (5×10^7 platelets/mL) were incubated with LM609 (20µg/mL) or modified Tyrode's buffer (control) for 20min at 37°C then allowed to adhere for 1h at 37°C. Adherent platelets were stained with TRITC-phalloidin and viewed under the ×60 objective of an IX71 fluorescence microscope (Olympus, Japan). Platelets from random visual fields with a total area of $0.1mm^2$ were counted. Data shown are means ± SD and are from 3 independent experiments with different blood donors. NS: not significant.

Since both $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ demonstrated partial involvement in mediating platelet adhesion to FXIIIa, their combined roles in the process were next investigated. Platelets were incubated with both tirofiban and LM609 prior to adhesion to FXIIIa. Combined inhibition of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ abolished platelet adhesion from 941±42 to 68±116 platelets/0.1mm^2 (P<0.01), reducing it to levels similar to those obtained with RGDS (103±90 platelets/0.1mm^2, P>0.05) (Figure 4.6). Substituting a non-reactive IgG control antibody for LM609 failed to enhance the inhibitory effects of tirofiban (619±115 vs 527±7 platelets/0.1mm^2 for tirofiban in the absence and presence of IgG respectively, P>0.05). This confirmed that the effects with LM609 were not due to non-specific antibody interactions with tirofiban or platelets. Together, these data suggest that maximal platelet adhesion to FXIIIa requires both $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins.

4.3 Platelet spreading on FXIIIa is dependent on $\alpha_{IIb}\beta_3$, but not $\alpha_v\beta_3$

Although both $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ mediated platelet adhesion to FXIIIa (Figures 4.2 and 4.4), adherent platelets displayed different morphologies when each receptor was blocked in isolation. Inhibition of $\alpha_{IIb}\beta_3$ ablated spreading responses in adherent platelets from 31±1μm^2 to 8±2μm^2 (P<0.01). In contrast, inhibition of $\alpha_v\beta_3$ did not affect platelet spreading (27±4μm^2 compared to 31±1μm^2 for the presence and absence of LM609 respectively, P>0.05) (Figure 4.7). Thus, platelet adhesion and spreading on FXIIIa are differentially mediated by $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$. While both receptors are required for adhesion, only $\alpha_{IIb}\beta_3$ is involved in the stimulation of spreading responses.

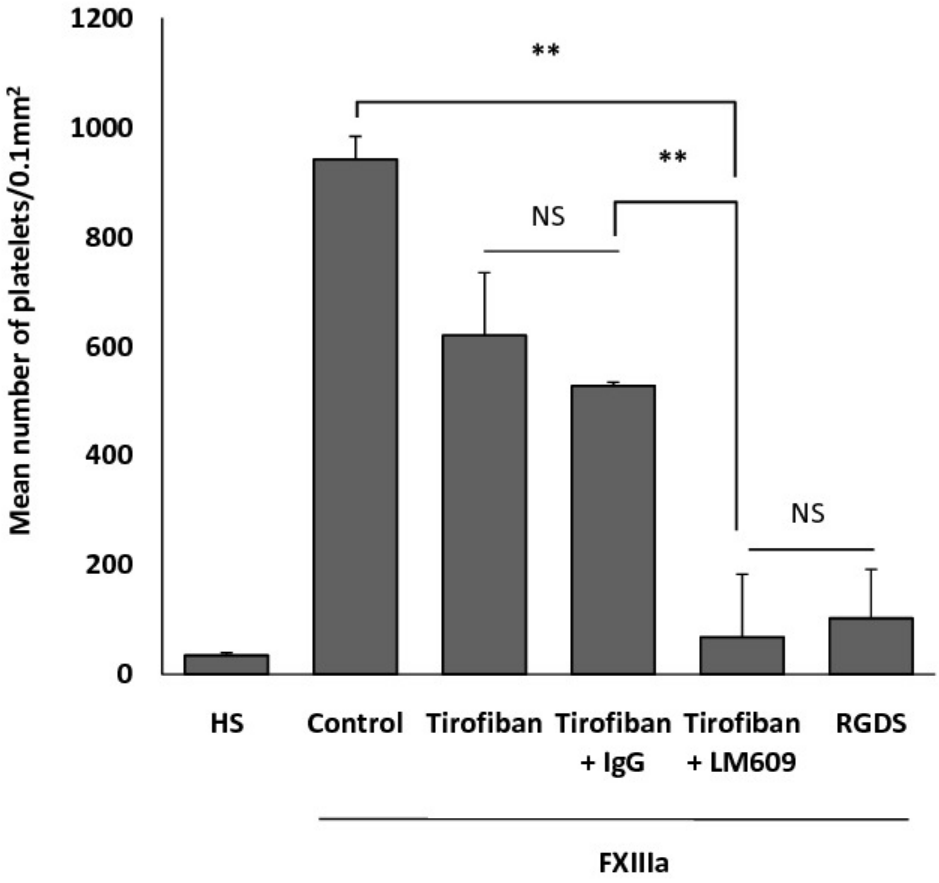
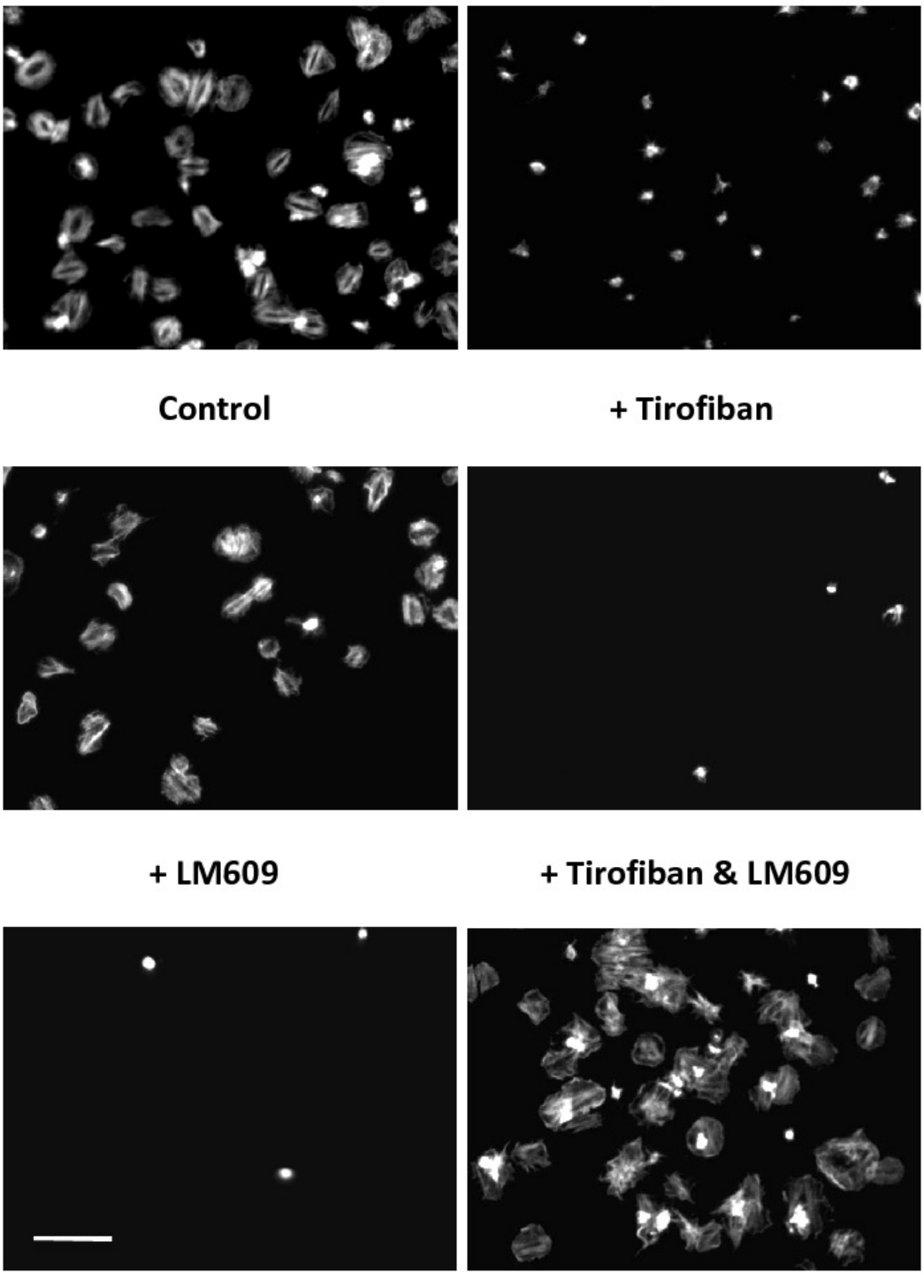


Figure 4.6: Platelet adhesion to FXIIIa is dually dependent on $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins. Wells on glass slides were coated overnight with heat-inactivated human serum (HS, 5%) or activated factor XIII (FXIIIa, 200µg/mL). Uncoated surfaces were blocked by overlay with HS (5%) for 30min at room temperature. Washed platelets (5×10^7 platelets/mL) were incubated with tirofiban (2µM), LM609 (20µg/mL), normal mouse IgG (20µg/mL), RGDS (500µM) or modified Tyrode's buffer (control) for 20min at 37°C then allowed to adhere for 1h at 37°C. Adherent platelets were stained with TRITC-phalloidin and viewed under the ×60 objective of an IX71 fluorescence microscope (Olympus, Japan). Platelets from random visual fields with a total area of $0.1mm^2$ were counted. Data shown are means ± SD and are from 3 independent experiments with different blood donors. NS: not significant, **: $P<0.01$.

(i)



(ii)

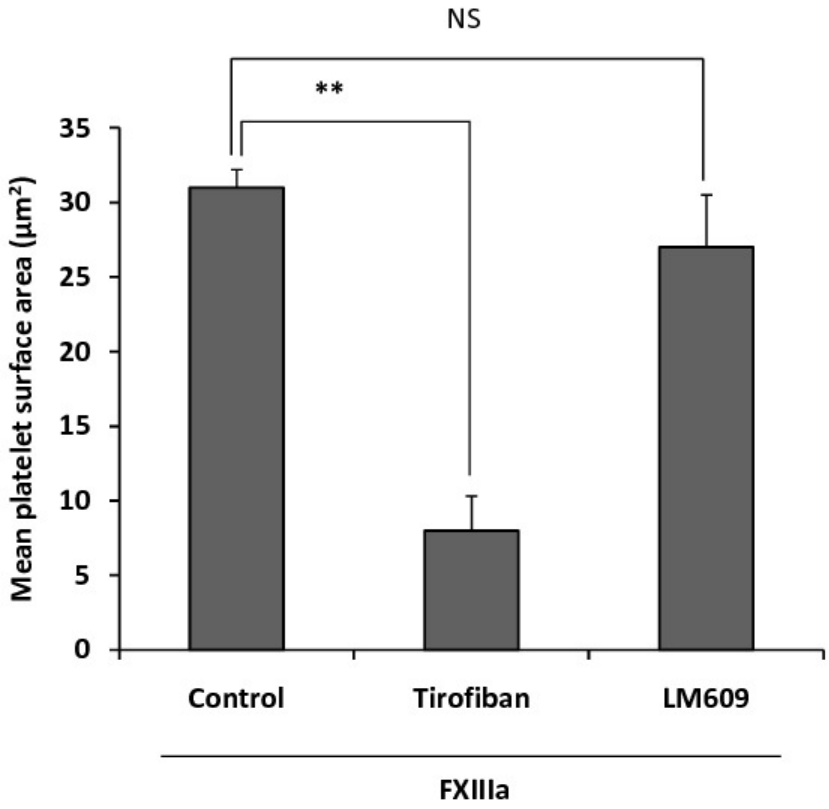


Figure 4.7: Platelet spreading on FXIIIa is mediated by $\alpha_{IIb}\beta_3$, but not $\alpha_v\beta_3$. Wells on glass slides were coated overnight with heat-inactivated human serum (HS, 5%) or activated factor XIII (FXIIIa, 200µg/mL). Uncoated surfaces were blocked by overlay with HS (5%) for 30min at room temperature. Washed platelets (5×10^7 platelets/mL) were incubated with tirofiban (2µM), LM609 (20µg/mL), normal mouse IgG (20µg/mL) or modified Tyrode's buffer (control) for 20min at 37°C then allowed to adhere for 1h at 37°C. Adherent platelets were stained with TRITC-phalloidin and viewed under the ×60 objective of an IX71 fluorescence microscope equipped with an XM10 CCD camera (Olympus, Japan). **(i)** Shown are representative images of adherent platelets captured under fluorescence. Scale Bar = 20µm. **(ii)** Surface areas covered by individual adherent platelets from random visual fields with a total area of $0.1mm^2$ were measured using ImageJ software (NIH, USA). Data shown are means ± SD from 3 independent experiments with different blood donors. NS: not significant, **: $P<0.01$.

4.4 Platelets adhere to FXIIIa under flow

The method of Cooke et al., (1993), was adapted to examine adhesive interactions between platelets and immobilised FXIII under physiological flow. Under this method, fluorescently stained washed platelets were reconstituted with autologous red blood cells and perfused through FXIII-coated glass microslide capillary tubes at various shear rates. Platelet adhesion and thrombus formation under these conditions were observed under fluorescent videomicroscopy and images of stably adherent platelets captured using a digital camera. Images were then used to calculate surface area coverage by adherent platelets as a measure of platelet adhesion.

4.4.1 Platelet adhesion to FXIII is shear-dependent

In the first instance, the ability of immobilised FXIII to support platelet adhesion under flow was assessed at $300s^{-1}$, a shear rate reported to represent venous blood flow using this methodology (Wu et al., 1997, Nesbitt et al., 2009). Under these conditions, video analysis revealed that platelet adhesion was characterised by rapid, immediate attachment with brief rolling in some instances to form an adherent platelet monolayer. Additional platelets were subsequently deposited over the monolayer leading to aggregate formation (Figure 4.8A and Video 1), suggesting activation and release of secondary agonists (ADP and TXA_2) by platelets in the monolayer (Ruggeri and Mendolicchio, 2007). Consistent with data obtained under static conditions (section 3.6.1), image analysis of platelets adherent under flow revealed similar surface coverage on zymogen and activated FXIII (6.2±0.4% vs 5.9±0.4% for FXIII and

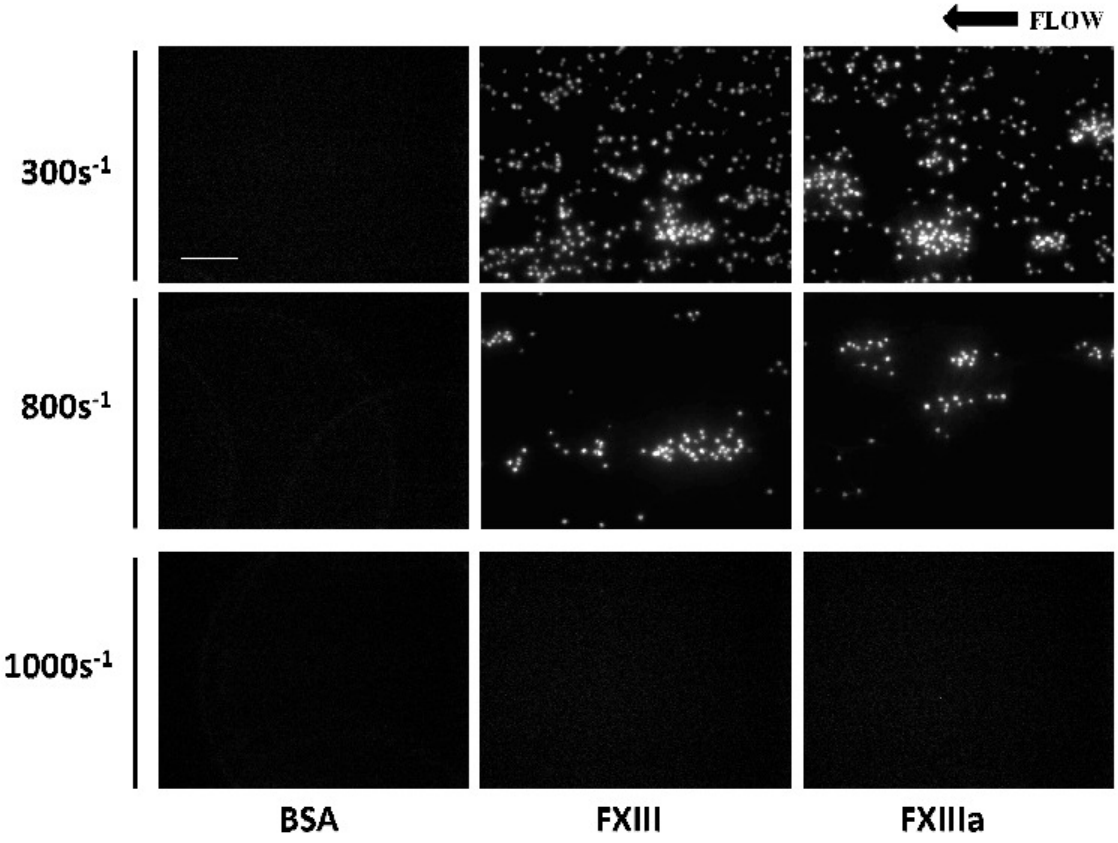
FXIIIa respectively $P>0.05$) (Figure 4.8A). Importantly, no adhesion was observed on BSA used to block platelet binding to uncoated glass, indicating that adhesive events were mediated by FXIII (Figure 4.8A).

Flow velocity was increased to generate an arterial shear rate of $800s^{-1}$, reported to be the threshold above which the presence of vWF is absolutely crucial for platelet adhesion to immobilised proteins under flow (Weiss et al., 1978b, Ruggeri, 2004, Ruggeri, 2007b). Although equal platelet arrest still occurred on both FXIII and FXIIIa (0.8±0.1% vs 0.9±0.1% surface coverage for FXIII and FXIIIIa respectively, $P>0.05$), in each case it was significantly less than at $300s^{-1}$ ($P<0.05$ for either FXIII or FXIIIa at $800s^{-1}$ vs $300s^{-1}$). However, it still caused significantly more surface coverage than BSA ($P<0.05$ vs FXIII and FXIIIa at $800s^{-1}$, ANOVA) (Figure 4.8A). Video analysis revealed that while many platelets tethered on the FXIII/FXIIIa surface at a shear rate of $800s^{-1}$, such associations were often weak and brief resulting in few stably adherent platelets with the majority being washed away in the direction of flow (Figure 4.8A and Video 2). No adhesive interactions were observable at shear rates above $1000s^{-1}$ (Figure 4.8A). Thus, immobilised FXIII and FXIIIa support equal platelet adhesion in a shear-dependent manner under physiological conditions of flow.

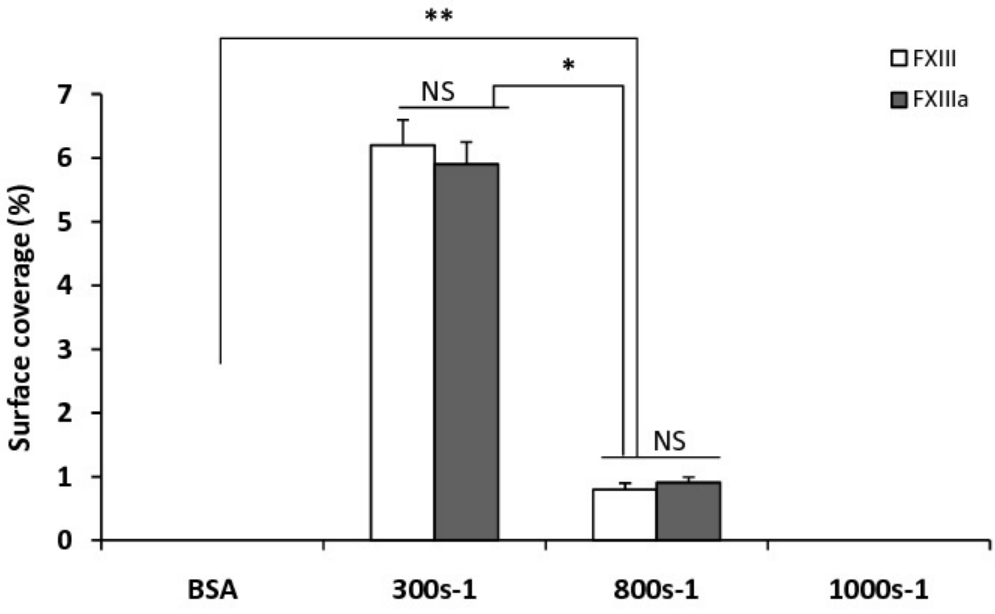
Since FXIII is a plasma transglutaminase, it associates with numerous plasma proteins (Muszbek et al., 1999). It was essential to ensure that the observed platelet adhesive interactions were indeed mediated by FXIII rather than an associated plasma protein. Hence, flow experiments were conducted with washed platelets and red blood cells for specific investigation (Figure 4.8A). However, it was important to ascertain whether the association

of platelets with FXIII was feasible in a more physiological environment of whole blood under flow. Therefore, whole blood was perfused through capillary tubes coated with either zymogen or activated rFXIII-A. In these experiments, rFXIII-A was used in order to exclude FXIII-B, which mediates FXIII-binding to fibrinogen (Siebenlist et al., 1996). Using whole blood, platelet adhesion to the enzyme was also observable under flow. Moreover, similar to data with washed platelets, adhesion proceeded equally on native and activated enzyme forms (6.1±0.3% and 6.5±0.6% surface coverage for rFXIII-A and rFXIIIa respectively, $P>0.05$) (Figure 4.8B). Nevertheless, these experiments could not be pursued due to the aforementioned difficulty of eliminating potential confounding effects of plasma proteins and the limited availability of rFXIII-A during the study.

A) (i)


FLOW
300s-1
800s-1
1000s-1
BSA
FXIII
FXIIIa

(ii)


**
*
NS
NS
FXIII
FXIIIa
Surface coverage (%)
0
1
2
3
4
5
6
7
BSA
300s-1
800s-1
1000s-1

B)

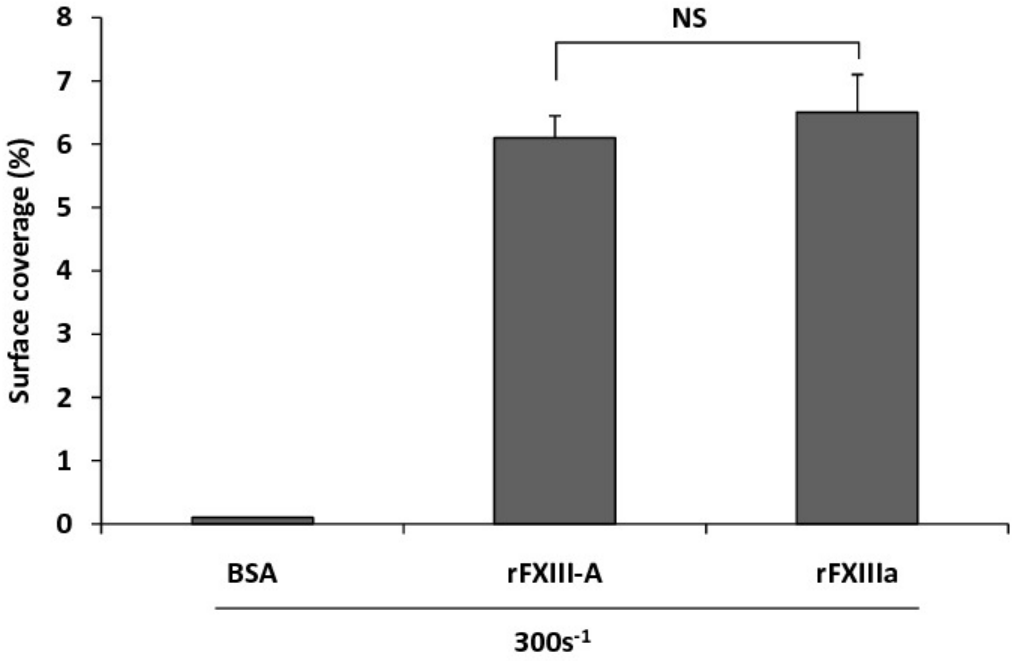


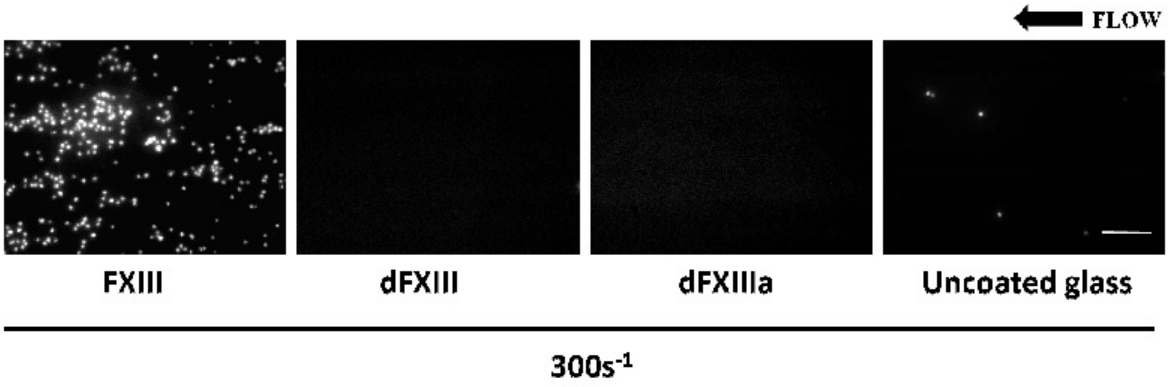
Figure 4.8: Platelets adhere equally to FXIII and FXIIIa under flow in a shear dependent manner. **(A)** Glass microslide capillary tubes were coated for 12 hours at 4°C with bovine serum albumin (BSA, 10mg/mL), zymogen factor XIII (FXIII, 200µg/mL) or activated factor XIII (FXIIIa, 200µg/mL). Uncoated surfaces were blocked by overlay with BSA (10mg/mL) for 1h at 20°C. Washed platelets were stained with $DiOC_6$ (1µM) and reconstituted with autologous red blood cells to final concentrations of 2×10^8 platelets/mL and 50% (v/v) respectively, then perfused through coated tubes for 4min at the indicated shear rate and platelet deposition viewed by fluorescence microscopy. Images from random visual fields with a total area of $0.1mm^2$ were captured under fluorescence using cell^P imaging software (Olympus, Japan). Magnification = ×60. **(i)** Shown are representative images. Scale bar = 20µm. **(ii)** Data are shown as percentage area coverage and are means ± SD of 5 independent experiments with separate blood donors. White bars (FXIII) and grey bars (FXIIIa). **(B)** As in **(A)**, except platelets were stained with $DiOC_6$ (1µM) in whole blood which was then perfused through capillary tubes that had been coated with either (BSA, 10mg/mL), zymogen recombinant factor XIII A-subunit (rFXIII-A, 200µg/mL) or activated rFXIII-A (rFXIIIa, 200µg/mL). **: $P<0.01$, *: $P<0.05$, NS: not significant.

4.4.2 Structure of FXIIIa influences platelet adhesion under flow

Under static conditions, the conformation of FXIII was observed to be crucial for platelet adhesion as evidenced by loss of platelet binding following heat-denaturation of the enzyme (section 3.8). To examine whether platelet adhesion similarly depended on protein structure under flow, FXIII was heat-denatured prior to immobilisation on microslide capillary tubes. Heat-treatment completely abolished platelet accrual on FXIII under flow ($P<0.01$). Similarly, no platelet adhesion could be observed on heat-denatured FXIIIa (Figure 4.9).

Under flow conditions, negligible platelet adhesion was observed on uncoated tubes which was significantly less than that occurring on FXIII-coated tubes (0.3±0.1 vs 6.2±0.4% surface coverage respectively, $P<0.05$). This provided evidence that observations attributed to platelet-FXIII interactions were not due to platelet adhesion on glass (Figure 4.9).

A (i)


FLOW
FXIII
dFXIII
dFXIIIa
Uncoated glass
300s-1

(ii)

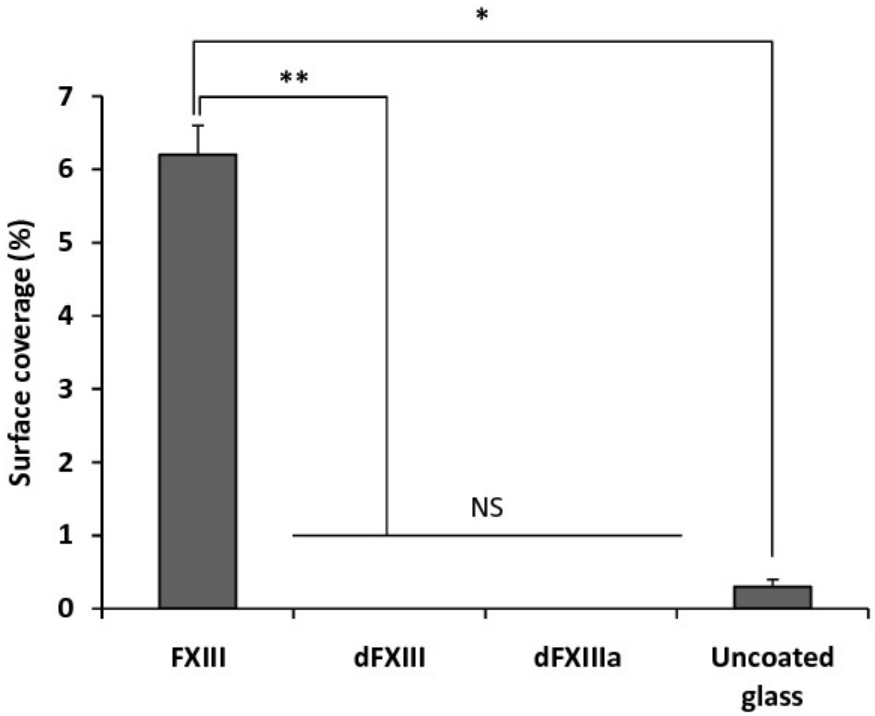

*
**
NS
Surface coverage (%)
0
1
2
3
4
5
6
7
FXIII
dFXIII
dFXIIIa
Uncoated glass

Figure 4.9: Protein structure of FXIII influences platelet adhesion under flow. **(A)** Factor XIII (FXIII, 200µg/mL) was used to coat glass microslide capillary tubes for 12hours at 4°C. In some cases, FXIII and activated FXIII (FXIIIa) were heat-denatured by incubation at 60°C for 1h to produce dFXIII (200µg/mL) and dFXIIIa (200µg/mL) respectively, which were also used for coating tubes. Uncoated surfaces were blocked with bovine serum albumin (10mg/mL) for 1h at room temperature. Washed platelets were stained with $DiOC_6$ (1µM) and reconstituted to a final concentration of 2×10^8 platelets/mL in autologous red blood cells (50%, v/v), then perfused through coated and uncoated glass tubes at a shear rate of $300s^{-1}$ for 4min and viewed by fluorescent microscopy. **(i)** Images from random visual fields with a total area of $0.1mm^2$ were captured under fluorescence using cell^P imaging software (Olympus, Japan). Magnification = ×60, scale bar = 20µm. **(ii)** Data are shown as percentage area coverage and are mean ± SD of 5 independent experiments with separate blood donors. **: $P<0.01$, *: $P<0.05$, NS: not significant.

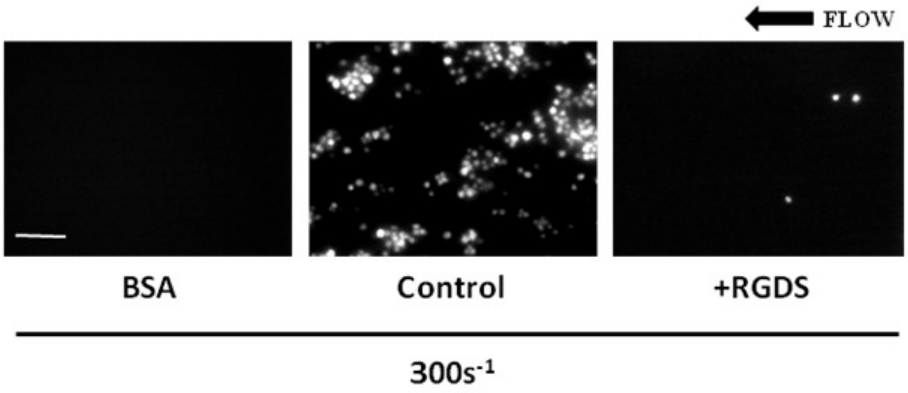
4.4.3 Platelet adhesion to FXIII under flow is dependent on integrins $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$

Data presented in section 4.2.2 suggested that FXIII-mediated platelet adhesion is dually dependent on $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins, with the two potentially playing complementary, non-redundant roles. The contributions of each receptor to adhesive interactions with FXIII were next examined under flow. Since activated FXIII predominates at sites of vascular injury due to high local thrombin concentrations (Troy, 1988, Narayanan, 1999), these experiments were performed with FXIIIa.

Consistent with experiments performed under static conditions (section 4.2.1), platelet adhesion under flow was abolished by RGDS (500μM) (5.7±0.6% vs 0.8±0.2% surface coverage for the absence and presence of RGDS respectively, $P<0.01$) (Figure 4.10).

Having confirmed the role of integrins under flow, receptor-specific inhibitors were then used to dissect the roles of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$. Incubation of platelets with tirofiban (2μM) significantly reduced platelet adhesion to FXIIIa under flow from 5.7±0.6% to 1.4±0.5% surface coverage (for the absence and presence of tirofiban respectively, $P<0.01$) (Figure 4.11). Similarly, when platelets were treated with LM609 (20μg/mL), surface coverage was reduced from 5.7±0.6% to 2.4±1.2% (for the absence and presence of LM609 respectively $P<0.01$). These data suggest that $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ play important roles in mediating adhesion to FXIIIa under flow.

(i)


FLOW
BSA
Control
+RGDS
300s-1

(ii)

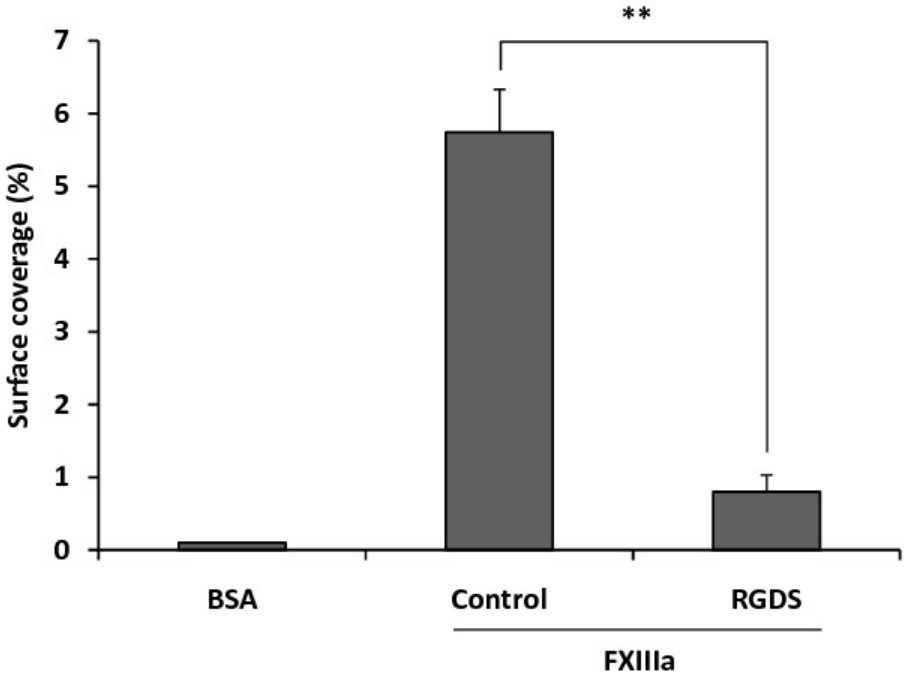
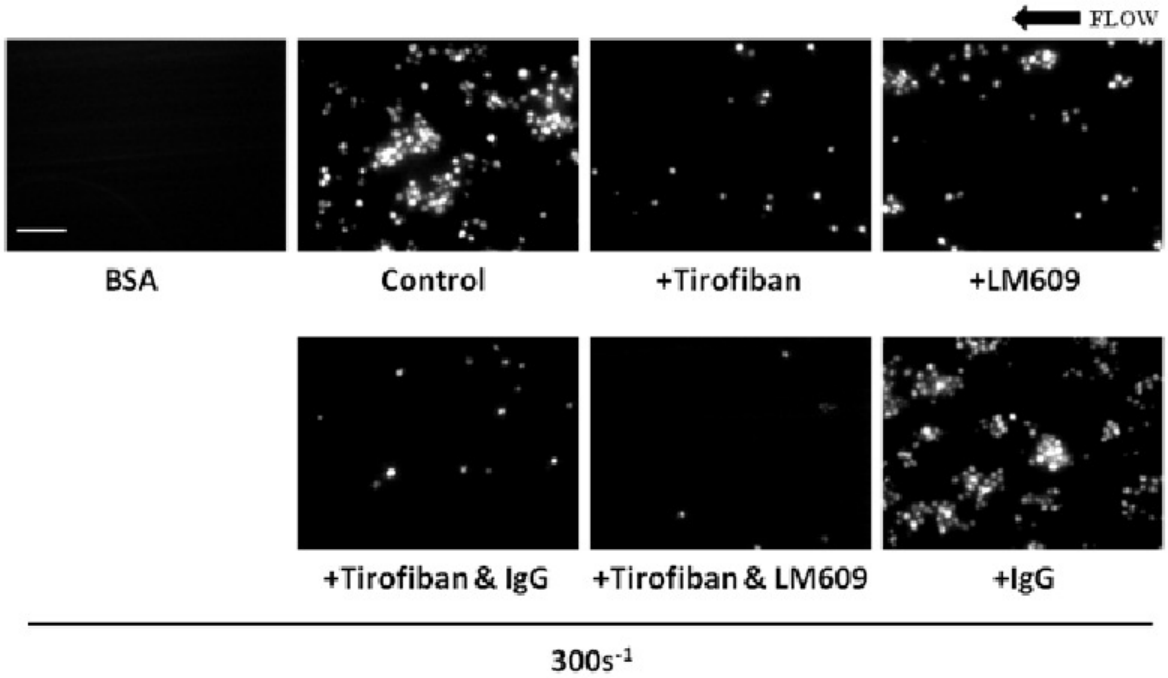

**
Surface coverage (%)
7
6
5
4
3
2
1
0
BSA
Control
RGDS
FXIIIa

Figure 4.10: Platelet adhesion to FXIIIa under flow is integrin-dependent. Washed platelets were treated with RGDS (500μM) to block integrins or modified Tyrode's buffer (control) for 20min at 37°C. They were stained with $DiOC_6$ (1μM) then reconstituted with autologous red blood cells to final concentrations of 2×10^8 platelets/mL and 50% (v/v) respectively and perfused through FXIIIa (200μg/mL)-coated capillary tubes for 4min at a shear rate of $300s^{-1}$. Thrombus formation was viewed using the ×60 oil-immersion lens of an IX71 fluorescence microscope equipped with an XM10 CCD digital camera (Olympus, Japan). **(i)** Shown are representative images of adherent platelets. Scale bar = 20μm. **(ii)** Images from random view fields with a total area of $0.1mm^2$ were used to calculate platelet surface area coverage (%) using ImageJ software (NIH, USA). Data are means ±SD from 4 separate experiments with different blood donors. *: $P<0.05$. BSA: bovine serum albumin.

Blocking both $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ simultaneously with tirofiban and LM609 ablated FXIIIa-mediated platelet adhesion reducing surface coverage from 5.7±0.6% to 0.3±0.2% (P<0.01) (Figure 4.11). Observations with LM609 could not be reproduced with a normal, isotype-matched IgG control antibody, thereby excluding potential non-specific antibody effects. Inhibition of $\alpha_{IIb}\beta_3$ reduced platelet adhesion significantly more than inhibition of $\alpha_v\beta_3$ (1.4±0.5% vs 2.4±1.2% for tirofiban and LM609 respectively, P<0.05), suggesting a more critical role for the former in mediating platelet adhesion under flow (Figure 4.11). Indeed, video analysis revealed that although platelet surface coverage was significantly diminished by separate inhibition of each integrin, tethering leading to stable contacts and aggregate formation was more dramatically reduced by $\alpha_{IIb}\beta_3$ blockade (Video 4). These findings reinforce data showing that platelet adhesion to FXIIIa is dually dependent on $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ while spreading that leads to stable adhesion is uniquely driven by $\alpha_{IIb}\beta_3$ (Section 4.3). In addition, while only single adherent platelets were observed following blockade of $\alpha_{IIb}\beta_3$, small platelet aggregates could still be seen when $\alpha_v\beta_3$ was inhibited in isolation (Figure 4.11Ai and Video 5). This is consistent with a recognised minor involvement of $\alpha_v\beta_3$ in platelet aggregation (Smith et al., 1990).

(i)


FLOW
BSA
Control
+Tirofiban
+LM609
+Tirofiban & IgG
+Tirofiban & LM609
+IgG
300s-1

(ii)

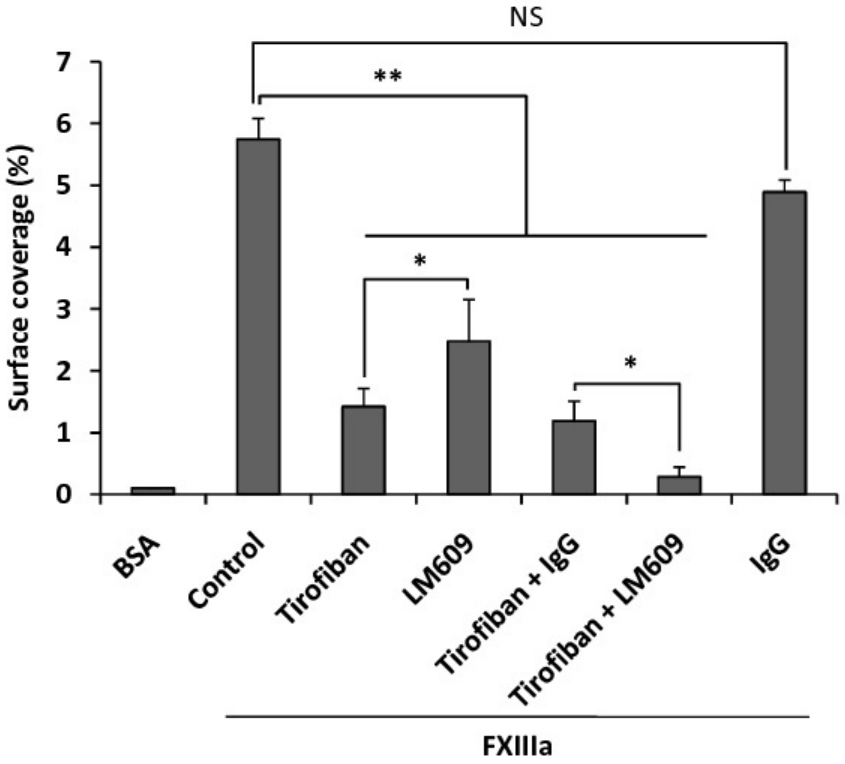

NS
**
*
*
Surface coverage (%)
0
1
2
3
4
5
6
7
BSA
Control
Tirofiban
LM609
Tirofiban + IgG
Tirofiban + LM609
IgG
FXIIIa

Figure 4.11: Platelet adhesion to FXIIIa under flow is dually dependent on $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$.
Washed platelets were treated with tirofiban (2μM), LM609 (20μg/mL), IgG control antibody (20μg/mL) or modified Tyrode's buffer (control) for 20min at 37°C. They were stained with $DiOC_6$ (1μM) then reconstituted with autologous red blood cells to final concentrations of 2×10^8 platelets/mL and 50% (v/v) respectively and perfused over immobilised FXIIIa (200μg/mL) for 4 minutes at a shear rate of $300s^{-1}$. Thrombus formation was viewed by fluorescence videomicroscopy. **(i)** Shown are representative images taken under ×60 magnification. Bar = 20μm **(ii)** Images from random fields of view with an area totalling $0.1mm^2$ were used to calculate platelet surface area coverage (%) using ImageJ software (NIH, USA). Data are means ± SD from 3 independent experiments with separate blood donors. **: $P<0.01$, *: $P<0.05$, NS: not significant, BSA: bovine serum albumin.

4.5 FXIII enhances platelet accrual by major thrombogenic platelet ligands under flow

At sites of vascular injury platelets bind to a plethora of adhesive proteins which influence their ability to form thrombi under flow (Ruggeri, 2002). Having observed that FXIII could recruit flowing platelets, the significance of these events in the context of adhesion to major platelet adhesive ligands was examined.

4.5.1 FXIII enhances platelet adhesion to fibrinogen under flow

FXIII can bind to fibrinogen in plasma where the two circulate in association (Greenberg and Shuman, 1982, Siebenlist et al., 1996). Since FXIII also binds platelets, its ability to influence platelet-adhesive properties of fibrinogen was investigated. To allow binding to fibrinogen, increasing concentrations of FXIII (0-200µg/mL) were incubated with a fixed concentration of fibrinogen (1mg/ml) prior to immobilisation and examination of platelet interactions under flow. Platelets were then either perfused over fibrinogen alone or in complex with FXIII.

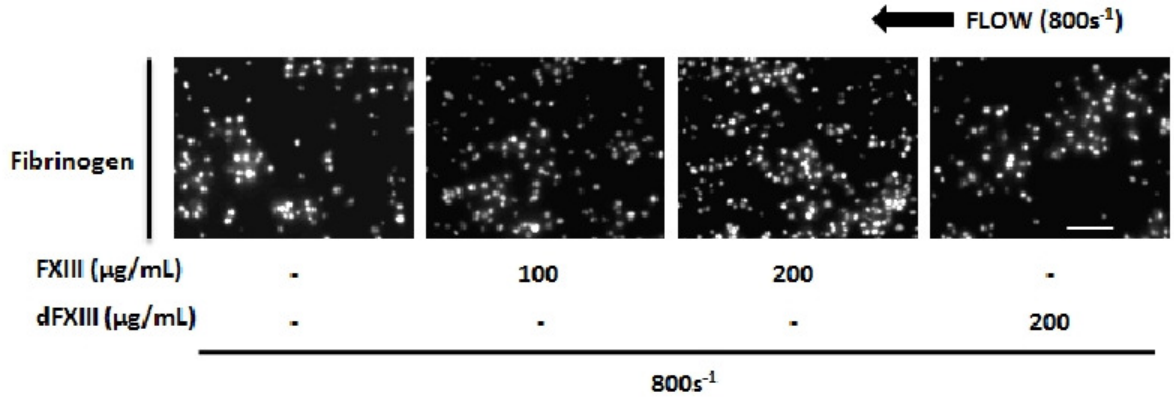
Fibrinogen alone supported the deposition of flowing platelets under an arterial shear rate of $800s^{-1}$ (9.6±0.9% surface coverage). However, preincubation with FXIII (100µg/mL) increased platelet recruitment by fibrinogen to 12.1±0.8% surface coverage ($P<0.05$) (Figure 4.12). This effect was not saturated at 200µg/mL of FXIII, the highest FXIII concentration used in these studies, since adhesion was further increased to 15.0±0.46% surface coverage ($P<0.01$ vs 100µg/mL FXIII). To exclude possible non-specific effects resulting from incubating fibrinogen with another protein, fibrinogen was incubated with heat-denatured FXIII (dFXIII, 200µg/mL)

prior to immobilisation. In contrast to findings with FXIII, dFXIII failed to enhance fibrinogen-mediated platelet adhesion (Figure 4.12). Effects of FXIIIa on fibrinogen-mediated platelet accrual could not be investigated since incubation of the two resulted in clotting by FXIIIa-mediated fibrinogen-chain cross-linking.

4.5.2 FXIIIa, but not FXIII binds to collagen and enhances collagen-mediated thrombus formation

FXIII is known to localise on collagen (Kikuchi et al., 1986, Blanchy et al., 1986). Moreover, it was reported to potentially mediate adhesion of bovine platelets to collagen (Kasahara et al., 1988). However, distinct abilities of FXIII and FXIIIa to bind collagen and influence collagen-mediated thrombus formation have not been assessed. FXIII and FXIIIa were suspended over collagen-coated glass surfaces. In each case, binding to collagen was examined using a primary antibody against FXIII-A and a fluorescent secondary antibody (section 2.5.2.3). The primary antibody's sensitivity for FXIII-A was demonstrated by its detection of immobilised FXIII (Figure 4.13), and rFXIII-A (Figure 3.2). Under these conditions, FXIIIa, but not FXIII bound to collagen (Figure 4.13). No signal was obtained from collagen or BSA used to block uncovered glass surfaces indicating that the primary antibody was specific for FXIII-A. Also, no signal was obtained from the secondary antibody in the absence of the primary antibody indicating that under these conditions, the former did not give false positive results by binding proteins non-specifically (Figure 4.13).

(i)


FLOW (800s-1)
Fibrinogen
FXIII (µg/mL) - 100 200 -
dFXIII (µg/mL) - - - 200
800s-1

(ii)

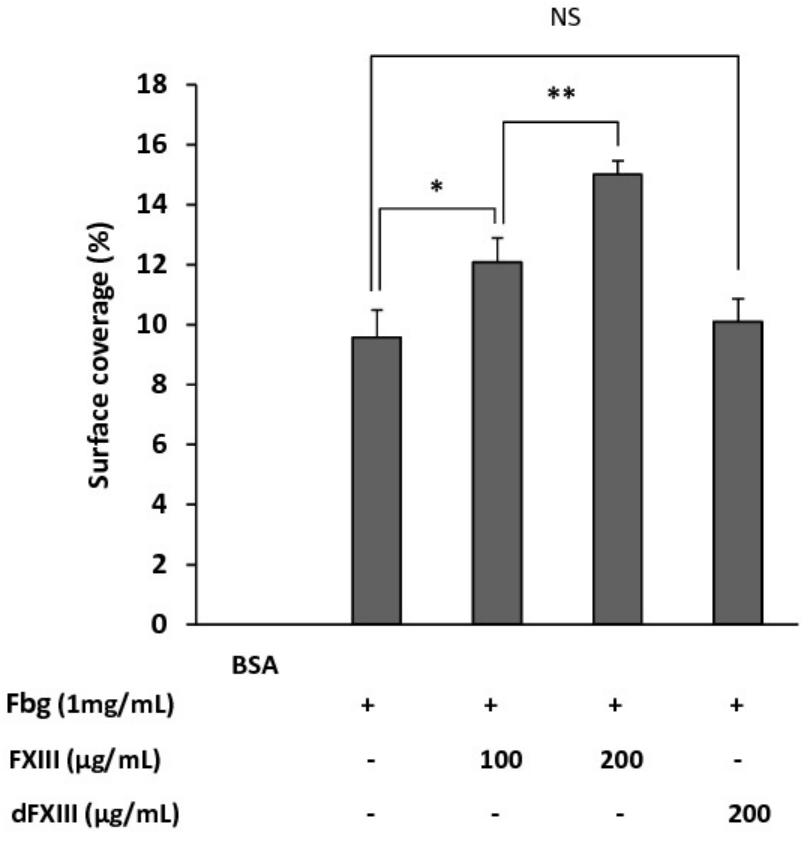

NS
**
*
Surface coverage (%)
18
16
14
12
10
8
6
4
2
0
BSA
Fbg (1mg/mL) + + + +
FXIII (µg/mL) - 100 200 -
dFXIII (µg/mL) - - - 200

Figure 4.12: Fibrinogen-bound FXIII enhances platelet binding to fibrinogen under flow. Zymogen factor XIII **(**FXIII, 0-200µg/mL) was allowed to bind to fibrinogen (Fbg, 1mg/mL) by 15min preincubation at 37°C prior to coating microslide capillary tubes. In some cases, FXIII was heat-denatured (dFXIII) by incubation at 60°C for 1h. Uncoated surfaces were blocked by overlay with bovine serum albumin (BSA, 10mg/mL) for 1h at 20°C. Washed platelets were stained with $DiOC_6$ (1µM) and reconstituted with autologous red blood cells to final respective concentrations of 2×10^8 platelets/mL and 50% (v/v) then perfused over the indicated surfaces at $800s^{-1}$ for 4min. **(i)** Shown are representative images captured under fluorescence videomicroscopy with the ×60 oil immersion lens of an IX71 fluorescence microscope (Olympus, Japan). Scale bar = 20µm. **(ii)** Images were used to calculate platelet surface area coverage. N=3 with separate blood donors. **: $P<0.01$, *: $P<0.05$, NS: not significant.

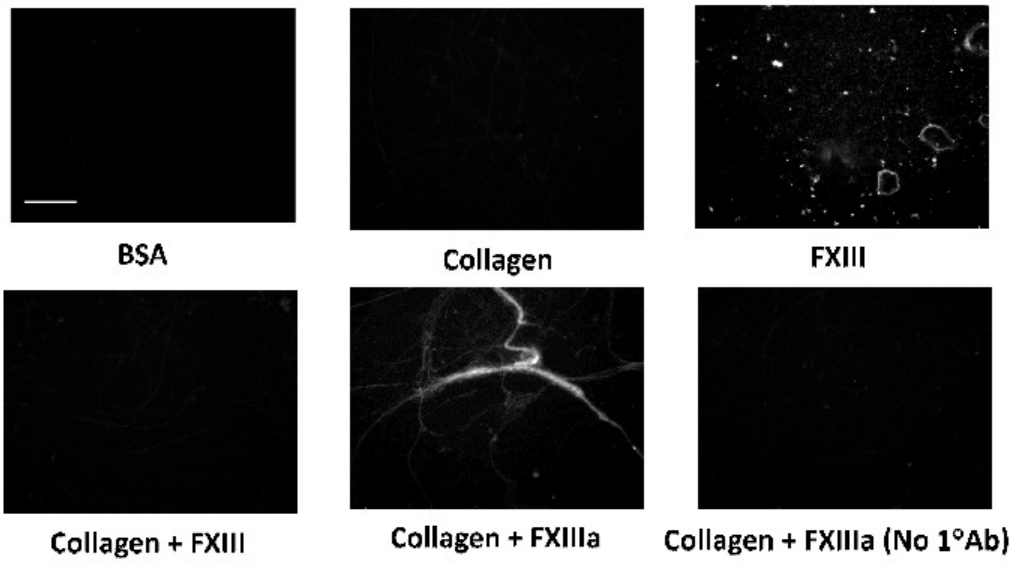
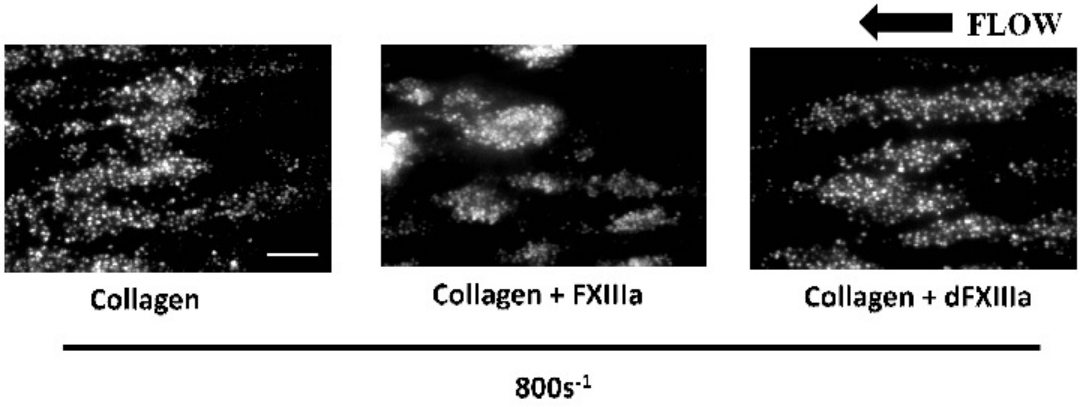


Figure 4.13: FXIIIa, but not FXIII immobilises on collagen. Wells on glass slides were coated overnight at 4°C with collagen (100µg/mL), bovine serum albumin (BSA, 10mg/mL) or factor XIII (FXIII, 200µg/mL). Uncoated surfaces were blocked with BSA (10mg/mL). Wells were then overlaid with zymogen FXIII (200µg/mL) or activated factor XIII (FXIIIa, 200µg/mL) for 15min at 37°C. Collagen-bound FXIII and FXIIIa were then detected by probing with a primary mouse antibody (1° Ab) against the A-subunit of factor XIII (FXIII-A). This was followed by incubation with a FITC-conjugated secondary goat anti-mouse antibody. Images were captured using the ×100 oil immersion lens of an IX71 fluorescence microscope (Olympus, Japan). Images are representative of 3 independent experiments. Bar = 20µm.

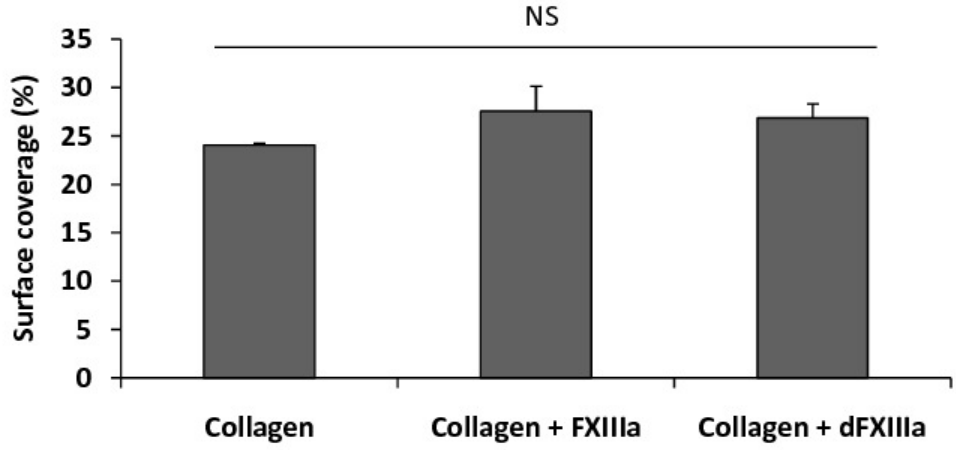
After establishing that FXIIIa was able to bind to collagen (Figure 4.13), and considering its platelet adhesive effects under flow (Figure 4.8), its role in collagen-mediated platelet accrual was next investigated. Washed platelets reconstituted with autologous red blood cells were perfused at $800s^{-1}$ over native collagen (100μg/mL) or collagen that had been preincubated with FXIIIa (200μg/mL) or dFXIIIa (200μg/mL) to allow binding. Under these conditions, collagen alone supported the deposition of robust platelet-rich thrombi which covered 24.0±0.2% of the surface. FXIIIa did not influence collagen-mediated platelet surface coverage which was maintained at 27.6±2.3% (P>0.05 vs collagen alone) (Figure 4.14ii).

However, visual image inspection revealed that thrombi formed in the presence of FXIIIa were more fluorescently intense (Figure 4.14i). Since fluorescence intensity can be used to determine thrombus volume (Ross et al., 1995), it was therefore measured. Using this approach (section 2.6.1), the mean thrombus volume on collagen alone ($3.7±0.1×10^5$ arbitrary units) was significantly increased by the presence of FXIIIa ($6.7±0.5×10^5$ arbitrary units, P<0.05 vs collagen alone) (Figure 4.14iii). Importantly, this required correct FXIIIa protein structure, since heat-denatured FXIIIa could not enhance the effects of collagen ($4.2±0.1×10^5$ arbitrary units P>0.05 vs collagen alone).

(i)


FLOW
Collagen
Collagen + FXIIIa
Collagen + dFXIIIa
800s-1

(ii)


NS
Surface coverage (%)
35
30
25
20
15
10
5
0
Collagen
Collagen + FXIIIa
Collagen + dFXIIIa

(iii)

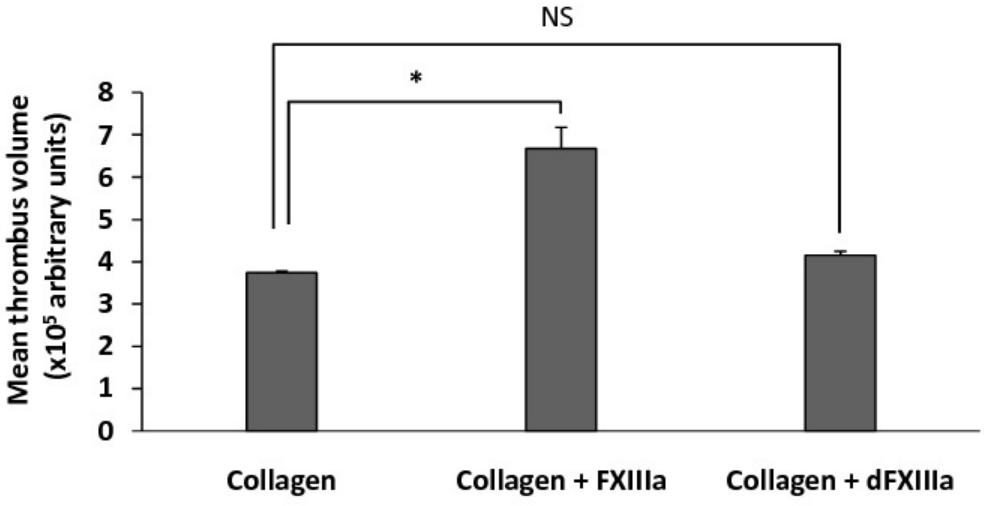

NS
*
Mean thrombus volume (x10⁵ arbitrary units)
8
7
6
5
4
3
2
1
0
Collagen
Collagen + FXIIIa
Collagen + dFXIIIa

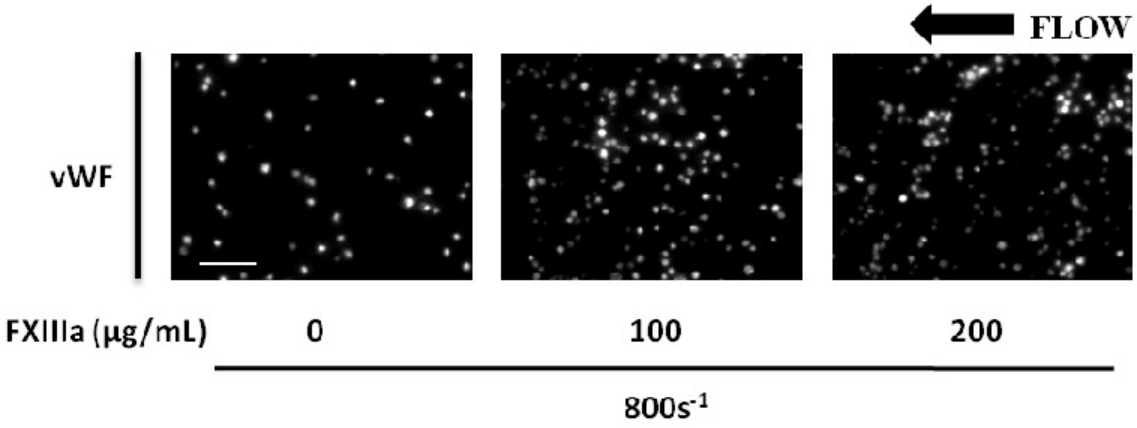
Figure 4.14: Collagen-bound FXIIIa accentuates platelet thrombus formation on collagen. Glass microslide capillary tubes were coated for 12 hours at 4°C with collagen (100µg/mL). In some cases FXIIIa (200µg/mL) or heat-denatured FXIIIa (dFXIIIa, 200µg /mL) were allowed to bind to collagen (100µg/mL) through 15min preincubation at 37°C prior to coating tubes. Uncoated surfaces were blocked by overlay with bovine serum albumin (BSA, 10mg/mL) for 1h at 20°C. Washed platelets were stained with $DiOC_6$ (1µM) and reconstituted with autologous red blood cells to final concentrations of (2×10^8 platelets/mL) and 50% (v/v) respectively, then perfused through coated tubes for 4min at a shear rate of $800s^{-1}$. Thrombus formation was viewed by fluorescence microscopy. **(i)** Shown are representative images of stable thrombi formed on each surface. Bar = 20µm **(ii)** ImageJ software (NIH, USA) was used to measure percentage surface area coverage. **(iii)** Volumes of thrombi formed on each surface were measured as previously described (Ross et al., 1995). All data shown are means ± SD from 4 separate experiments with different blood donors. *: $P<0.05$, NS: not significant.

4.5.3 FXIIIa accentuates platelet recruitment by vWF at high shear

vWF is critical for platelet recruitment to vascular injury sites at elevated shear (Ruggeri and Ware, 1993). However its interaction with platelets through GP1b/IX/V is transient and platelets require integrin-mediated ligand engagement for stable adhesion (Jackson, 2007). Since FXIIIa adhered to platelet integrins under flow (section 4.4.3), its ability to supplement platelet recruitment by vWF was investigated.

At a shear rate of $800s^{-1}$, immobilised vWF (20µg/mL) supported transient tethering and rolling of flowing platelets with only single platelets adhering stably to cover 8.9±0.1% of the vWF-coated surface (Figure 4.15). However, platelet surface coverage was significantly increased when vWF was co-immobilised with FXIIIa (100µg/mL) (14.3±3.8%, $P<0.05$ vs vWF alone) (Figure 4.15ii). Increasing the concentration of FXIIIa to 200µg/mL did not further enhance platelet recruitment since surface coverage was insignificantly changed (15.0±1.6%, $P>0.05$ vs FXIIIa at 100µg/mL). Importantly, there was clear contrast between single platelets adherent on vWF and platelet aggregates observed on vWF/FXIIIa (Figure 4.15i). These data indicate that FXIIIa can enhance vWF-mediated platelet accrual at high shear. Furthermore, the presence of vWF facilitates greater platelet deposition on immobilised FXIIIa at arterial shear since platelet surface coverage was increased from 0.9±0.1% to 15.0±1.6% ($P<0.01$ for the absence and presence of vWF respectively) (Figure 4.15ii).

(i)


FLOW
vWF
FXIIIa (µg/mL)
0
100
200
800s-1

(ii)

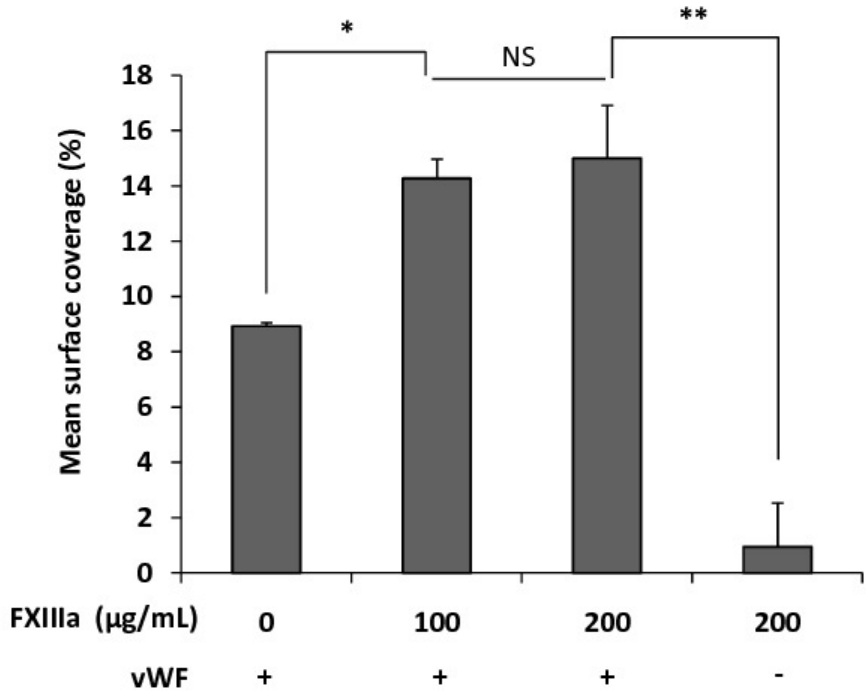

*
NS
**
Mean surface coverage (%)
18
16
14
12
10
8
6
4
2
0
FXIIIa (µg/mL)
0
100
200
200
vWF
+
+
+
-

Figure 4.15: FXIIIa enhances platelet accrual by vWF at arterial shear. Washed platelets were stained with $DiOC_6$ (1µmol/L) and reconstituted with autologous red blood cells to final concentrations of 2×10^8 platelets/mL and 50% (v/v) respectively. This was perfused over immobilised FXIIIa (10U/mL) or vWF (20µg/mL) either alone or co-immobilised with increasing concentrations of FXIIIa (20-200µg/mL) for 4 minutes at a shear rate of $800s^{-1}$ and viewed by fluorescence microscopy. Images from random fields of view covering a total area of $0.1mm^2$ were captured under fluorescence using cell^P imaging software from Olympus (magnification = ×60). **(i)** Shown are representative images. Scale bar = 20µm **(ii)** Data shown are means ± SD of 3 separate experiments with different blood donors. **: $P<0.01$. *: $P<0.05$.

4.6 Discussion

The present study concentrated on examining platelet adhesion to immobilised FXIII and investigating the ensuing effects on platelet function. Having confirmed the purity of the source of FXIII used in these studies from contaminating platelet adhesive plasma proteins (section 3.2), characterisation of the platelet receptors mediating adhesion to FXIII was performed.

The essential role of integrin receptors in platelet adhesion to immobilised FXIII was demonstrated by its abolition when platelet integrins were broadly inhibited by RGDS (Figure 4.1). Given that previous studies had reported a role for $\alpha_{IIb}\beta_3$ in mediating platelet adhesion to suspended FXIII (Cox and Devine, 1994, Dardik et al., 2002b), its role in binding the immobilised enzyme was assessed. Specific inhibition of $\alpha_{IIb}\beta_3$ with tirofiban partially, but significantly reduced platelet adhesion (Figure 4.2). This was indicative of its importance in these events, but suggested the involvement of other integrin receptors. Partial inhibition of platelet adhesion to FXIII under these conditions was not due to incomplete receptor inhibition since tirofiban at the same concentration ablated adhesion to fibrinogen (Figure 4.3), which is known to be critically dependent on $\alpha_{IIb}\beta_3$ integrins (Bennett et al., 1983, French and Seligsohn, 2000).

Further investigation revealed that $\alpha_v\beta_3$ supplemented $\alpha_{IIb}\beta_3$-mediated platelet adhesion to FXIII. Adhesion was also partially, but significantly dependent on $\alpha_v\beta_3$ and was abolished only when both receptors were blocked (Figure 4.6), indicating that they collaborated in these

events. This ablation of adhesion suggests that platelets do not possess additional receptors for FXIII.

In attempt to specifically demonstrate the crucial role of $\alpha_{IIb}\beta_3$ in mediating platelet adhesion to FXIII, previous studies used the antibody 7E3 (Cox and Devine, 1994), its humanised derivative abciximab (Dardik et al., 2002b) or eptifibatide (Nagy et al., 2009). However, all three inhibitors also block $\alpha_v\beta_3$, unlike tirofiban which is strictly selective for $\alpha_{IIb}\beta_3$ (Nurden et al., 1999, Lele et al., 2001). Thus, these studies overlooked the potential involvement of $\alpha_v\beta_3$ in adhesive events. Identification of $\alpha_v\beta_3$ involvement in FXIII-mediated platelet binding therefore constitutes a novel finding of a potential platelet FXIII-receptor.

Estimates of $\alpha_v\beta_3$ copy number on the platelet surface range from 1485 ± 780 (mean ± SD) (Lawler and Hynes, 1989) to 50-100 (Coller et al., 1991). This makes its surface expression considerably lower than that of $\alpha_{IIb}\beta_3$ which is estimated to be about 80000 per platelet (Wagner et al., 1996). However, it is noteworthy that platelet $\alpha_v\beta_3$ has been shown to possess an affinity 150 times greater than $\alpha_{IIb}\beta_3$ for RGD sequences (Smith et al., 1990). In fact, although platelet adhesion to vitronectin is jointly mediated by $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins, $\alpha_v\beta_3$ is responsible for as much as 26% of the adhesive interactions (Coller et al., 1991). Thus, despite its lower copy number $\alpha_v\beta_3$ is an important mediator of platelet adhesive events.

Interestingly, separate studies using platelets from patients with GT gave conflicting results regarding the role of $\alpha_{IIb}\beta_3$ in platelet-FXIIIa interactions. Using platelets from a single patient, Cox and Devine (1994) reported severely diminished FXIIIa-binding whereas

Greenberg and Shuman (1984) observed normal platelet-FXIIIa binding by platelets from two patients. While the two studies are not directly comparable due to differences in methodology, the conflicting findings may also be due to differences in receptor defects among patients with GT. For instance, it is known that in GT sufferers with mutations in β_3 chains, both $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ have functional defects, whereas when α_{IIb} chains are affected; $\alpha_v\beta_3$, but not $\alpha_{IIb}\beta_3$ may retain normal function (Nurden, 2006). In order to resolve discrepancies among reports, future studies with well-characterised platelets with defective α_{IIb} chains will need to be performed. However, the fact that platelets from patients with GT can still adhere to vitronectin through $\alpha_v\beta_3$ (Coller et al., 1991), and the ability of this receptor to also mediate FXIII(a)-binding by endothelial cells (Dallabrida et al., 2000, Dardik et al., 2002a), monocytes (Dardik et al., 2007) and fibroblasts (Ueki et al., 1996) together support its involvement in the platelet-FXIII(a) interactions reported in the present study.

Visual microscopic analysis of FXIIIa-adherent platelets revealed that although separate inhibition of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins reduced platelet adhesion (Figures 4.2 and 4.4), the treatments had different effects on adherent-platelet morphology. While unaffected by inhibition of $\alpha_v\beta_3$, platelet spreading responses were abolished following $\alpha_{IIb}\beta_3$ blockade (Figure 4.7). This suggests that the two receptors mediate different, non-redundant functional effects in adherent platelets. Together, these data suggest a model where $\alpha_{IIb}\beta_3$ complements $\alpha_v\beta_3$-mediated platelet adhesion to FXIIIa through stimulation of platelet spreading. Indeed, spreading is an activation-dependent response that allows adherent platelets to cover larger surface areas and adhere more stably (Jen et al., 1996, Kuwahara et al., 2002).

Although adhesive interactions between platelets and suspended FXIII are recognised (Greenberg and Shuman, 1984, Devine et al., 1993, Cox and Devine, 1994, Nagy et al., 2009), they have not been studied under physiological flow conditions nor have their potential contributions to platelet accrual by extracellular matrix proteins exposed at vascular injury sites been investigated. Both immobilised FXIII and FXIIIa were observed to equally support platelet adhesion under flow at venous and arterial shear rates (Figure 4.8). This was consistent with data obtained under static conditions where platelet adhesion occurred independently of transglutaminase activity (section 3.7.1). However, platelet adhesion to both FXIII and FXIIIa was influenced by prevailing flow conditions. At venous shear, platelets were readily recruited and adhered stably to form aggregates, while at arterial shear, platelet adhesion was markedly reduced, but not abolished (Figure 4.8). This was in line with the formation of integrin-mediated platelet contacts which are known to occur too slowly to facilitate platelet-binding to integrin ligands including fibrinogen, fibronectin and vitronectin under rapid arterial flow in the absence of vWF (Zaidi et al., 1996). Indeed, platelet adhesion to FXIIIa at arterial shear was significantly increased in the presence of vWF (Figure 4.15ii). Thus, similar to other integrin ligands, platelet adhesion to FXIIIa under flow is shear-dependent, diminished at high shear where it is critically dependent on vWF which retards platelets to allow stable adhesive contacts to form.

Under flow, platelet adhesion to FXIIIa was also observed to be dually dependent on $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins since it was only effectively abolished when both integrins were blocked (Figure 4.11). Consistent with data obtained under static conditions (Figure 4.7), separate

inhibition of each integrin had different effects on adhesion under flow. Although blockade of $\alpha_v\beta_3$ significantly reduced adhesion, analysis of platelets flowing at venous shear revealed that they were still able to form stable contacts with no increase in transient tethering and rolling observed (Figure 4.8i and Video 5). In contrast, blockade of $\alpha_{IIb}\beta_3$ more severely limited the ability of platelets to form stable contacts or aggregate resulting in a greater tendency to disengage from FXIIIa and wash away in the direction of flow (Figure 4.8i and Video 4). This suggests that spreading occurring downstream of $\alpha_{IIb}\beta_3$ was required to stabilise adhesive contacts with FXIIIa. Therefore, while both $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ integrins are involved in facilitating the initial platelet engagement with FXIII, stable adhesion is critically dependent on $\alpha_{IIb}\beta_3$.

FXIII associates with multiple thrombogenic platelet ligands (Muszbek et al., 1999). Since FXIII also supported platelet recruitment under flow, it was possible that it could increase thrombus formation by key platelet ligands. Collagen-bound vWF mediates platelet tethering, retarding flowing platelets and allowing their activation in response to exposed matrix proteins at high shear (Ruggeri and Ware, 1993). Firm platelet adhesion then takes place which is strengthened by spreading following integrin-mediated binding of various plasma proteins (Shattil and Newman, 2004). Although solely immobilised vWF captured single platelets at high shear, the presence of FXIIIa increased platelet accrual as evidenced by enhanced platelet surface coverage and aggregate formation, which was unobservable on vWF alone (Figure 4.15i). Hence, FXIIIa may act in concert with vWF to arrest platelets flowing at high shear.

FXIIIa, but not FXIII bound to collagen and enhanced the ability of this key matrix protein to form platelet-rich thrombi (Figures 4.13 and 4.14). Thus, although activation of the enzyme is not essential for its adhesive interactions with platelets (section 3.7.1), activation allows FXIIIa to localise on exposed collagen fibres. This may specifically recruit FXIIIa to the ECM where through its cross-linking reactions that reorganise matrix proteins, it facilitates fibroblast migration and wound healing (Brown et al., 1993, Inbal et al., 2005). Despite collagen being the most potent platelet agonist in the ECM, its ability to bind platelets through GPVI and $\alpha_2\beta_1$ receptors is limited by the low affinity of GPVI and low copy number of $\alpha_2\beta_1$ (Watson et al., 2005). Hence, by binding collagen exposed at vascular injury sites; FXIIIa like vWF may increase platelet recruitment by supplementing platelet-binding sites available per collagen fibre. This may explain why platelet surface coverage was not increased by collagen-bound FXIIIa (Figure 4.14ii), since its adhesive interactions with platelets were likely confined to where it was physically associated with each collagen fibre.

Fibrinogen becomes localised on the ECM following vascular injury where it is thrombin-cleaved to form fibrin clots (Duckert and Nyman, 1978). In plasma, FXIII can associate with fibrinogen (Siebenlist et al., 1996), a ligand for $\alpha_{IIb}\beta_3$ and a vital mediator of platelet aggregation (Jackson, 2007). Since fibrinogen is present in excess of FXIII in plasma, much of the enzyme potentially circulates bound to fibrinogen (Greenberg and Shuman, 1982). Platelet adhesion to plasma protein-free recombinant FXIII-A (Figure 3.6) and the absence of fibrinogen from Fibrogammin P (section 3.2) strongly suggested that platelets were able to adhere to FXIII independently of fibrinogen. Consistent with a previous report (Smith et al., 1990), sole blockade of $\alpha_v\beta_3$ had no measurable effect on platelet adhesion to fibrinogen

(Figure 4.5). This supported data showing fibrinogen-independent adhesion since platelet adhesion to FXIII would not have been expected to decrease following treatment with LM609 (Figure 4.4), if it were mediated by fibrinogen. Crucially, it also suggested that different, non-redundant mechanisms controlled platelet-binding to the two proteins. Indeed, when bound to immobilised fibrinogen, FXIII dose-dependently increased fibrinogen-mediated platelet recruitment under flow (Figure 4.12), suggesting that it may collaborate with fibrinogen in mediating platelet accrual.

These data appear to contradict a recent report suggesting that FXIII does not bind platelets directly, but instead relies on its association with fibrinogen to interact with platelets (Nagy et al., 2009). However, the data are supported by findings from Cox and Devine (1994) who demonstrated FXIII-binding by washed platelets and isolated $\alpha_{IIb}\beta_3$ integrins in the absence of fibrinogen which enhanced the process when present. In any case, both studies examined platelet-FXIII associations occurring in suspenstion using flow cytometry while platelet interactions with the immobilised enzyme were considered in the present work. Thus methodological differences limit comparability of results.

Intravital and *in vitro* perfusion studies examining thrombus formation in genetically modified mice have revealed important roles for plasma proteins outside the recognised major platelet adhesive ligands in thrombus formation. Using mice deficient in vWF and fibrinogen, Ni et al., (2000) demonstrated that although considerably incapacitated, the mice were still able to form occlusive platelet thrombi in response to vascular injury, even at elevated shear. As a result, an important role for fibronectin in thrombus formation under *in*

vivo flow was established (Ni et al., 2003). More recently, mice deficient in all three proteins (vWF, fibrinogen and fibronectin) were also observed to retain an ability to form platelet thrombi under flow (Reheman et al., 2009). Thus, *in vivo* thrombus formation involves complex interactions between platelets and multiple plasma proteins potentially including FXIII. Indeed, FXIII-deficient mice exhibit elongated tail bleeding times (Lauer et al., 2002) and markedly diminished clot retraction (Kasahara et al., 2010), suggesting a potential role for FXIII in platelet-dependent thrombosis. Hence, the present data add FXIII to the growing list of proteins known to localise at sites of vascular damage and synergistically activating blood platelets through manifold integrated signalling pathways.

In summary, this chapter has further clarified the nature of physical interactions between platelets and FXIII by characterising the roles of individual receptors involved. Moreover, the potential implications of adhesion events with respect to other platelet adhesive proteins under physiological conditions of flowing blood have been discussed. This provides a platform to understand the signalling events initiated downstream of receptor occupation and how they lead to platelet functional responses which will be addressed in the next chapter.

CHAPTER 5 - ADHESION TO FXIII ACTIVATES AN INTEGRIN-MEDIATED SIGNALLING CASCADE IN PLATELETS.

5.1 Introduction

Blood platelets circulate in quiescent states and fulfil their haemostatic roles following restricted activation at sites of vascular injury. Platelet activation is induced by an array of agonists which stimulate signal transduction after binding to specific platelet-surface receptors (Rivera et al., 2009). The ensuing signalling events transform physical receptor occupation at the platelet surface to functional responses by relaying activatory information through intracellular biochemical signalling networks.

Although activatory signals may lead to platelet responses including spreading, secretion and expression of procoagulant phosphatidylserine on the membrane surface (Zucker and Nachmias, 1985), the transformation of platelet integrin receptors to their high affinity states is perhaps most important for platelet clot formation. Indeed, this "final common pathway" of platelet activation facilitates platelet thrombus formation through fibrinogen binding by $\alpha_{IIb}\beta_3$ integrins (Ruggeri, 2002). However, far from being passive mediators in clot formation, integrins also transmit activatory signals upon binding their ligands (Giancotti and Ruoslahti, 1999). This "outside-in" signalling perpetuates and reinforces clot formation, essentially making platelet activation a process dependent on bidirectional signalling (Shattil et al., 1998).

In Chapter 3, data demonstrating spreading and secretory responses by FXIII-adherent platelets were presented. The characteristic display of such responses by activated platelets and the known involvement of $\alpha_{IIb}\beta_3$ in mediating platelet adhesion to FXIII guided a hypothesis that FXIII stimulates activatory signalling in adherent platelets.

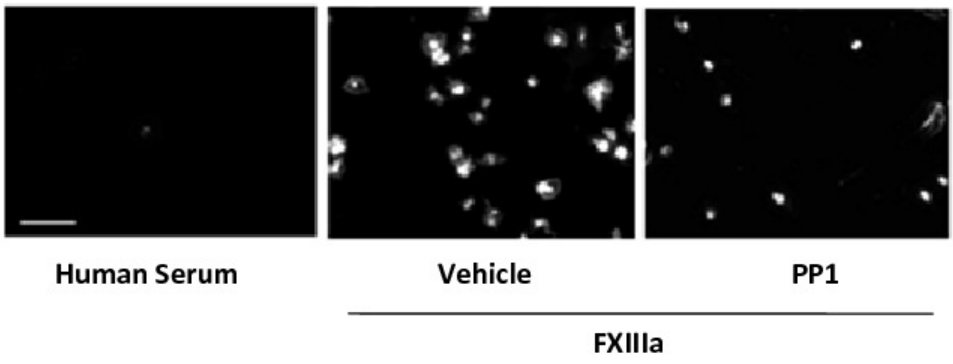
Aims:

- To examine whether biochemical signal-transduction occurs in FXIII-adherent platelets and if so, to identify some of the signalling molecules involved.
- To establish the relative roles of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ in mediating the signalling events.
- To determine how platelet activation influences adhesive contacts with FXIII.

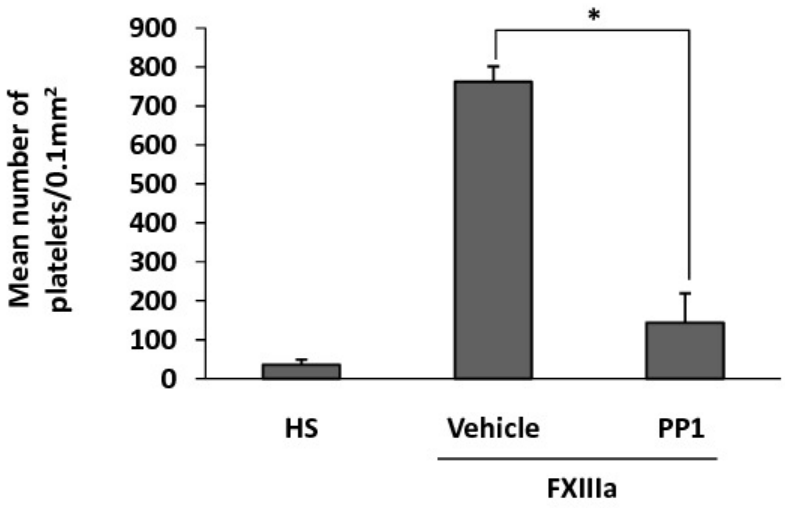
5.2 Platelet-FXIIIa interactions require Src-family tyrosine-kinases

Src-family kinases are crucially involved in initiating signal transduction downstream of $\alpha_{IIb}\beta_3$ integrins following occupancy by their ligands (Gao et al., 1997, Obergfell et al., 2002). Since $\alpha_{IIb}\beta_3$ integrins facilitated FXIIIa-mediated platelet adhesion and spreading (section 4.3), the involvement of Src-family kinases in these events was assessed. Incubating platelets with PP1 (20μM), a selective Src-family kinase inhibitor (Hanke et al., 1996), significantly attenuated spreading on FXIIIa (27±1 to 11±3μm^2 for the absence and presence of PP1 respectively, $P<0.01$). Furthermore, stable platelet adhesion was also diminished (762±40 to 143±77 platelets/0.1mm^2 for the absence and presence of PP1 respectively, $P<0.05$) (Figure 5.1).

(i)


Human Serum
Vehicle
PP1
FXIIIa

(ii)


Mean number of platelets/0.1mm²
900
800
700
600
500
400
300
200
100
0
*
HS
Vehicle
PP1
FXIIIa

(iii)

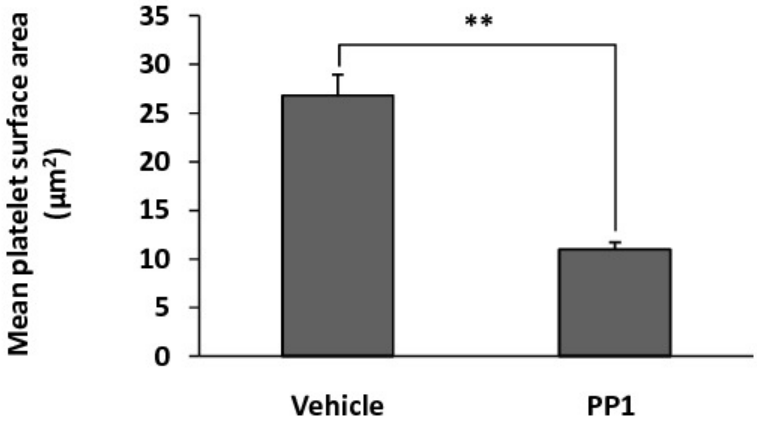
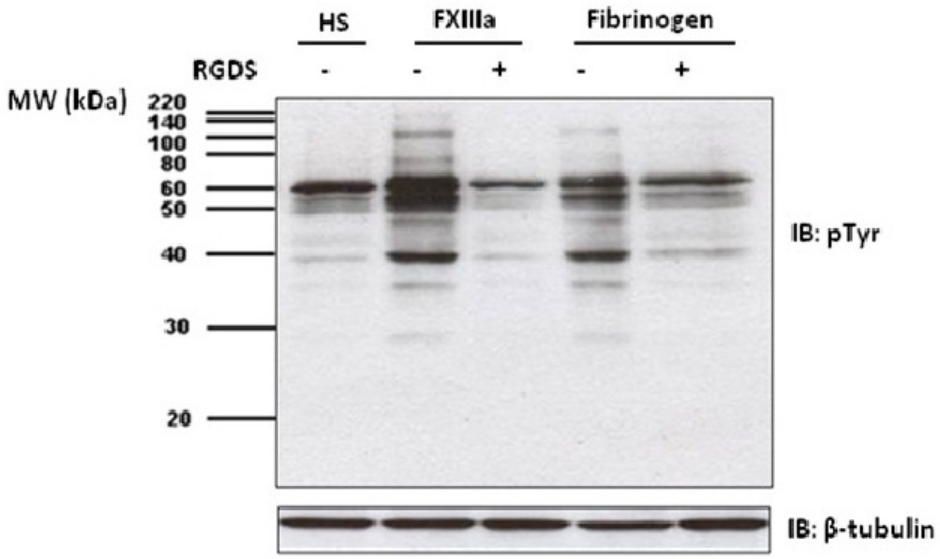

Mean platelet surface area (μm²)
35
30
25
20
15
10
5
0
**
Vehicle
PP1

Figure 5.1: Platelet adhesion and spreading on FXIIIa is tyrosine-kinase dependent. Wells on glass slides were coated overnight with heat-inactivated human serum (HS, 5%) or activated factor XIII (FXIIIa, 200µg/mL). Uncoated surfaces were blocked by overlay with HS (5%) for 30min at room temperature. Washed platelets (5×10^7 platelets/mL) were treated with PP1 (20µM) or DMSO (0.01%, vehicle control) for 20min at 37°C then adhered for 1h at 37°C. Adherent platelets were viewed using an IX71 fluorescence microscope equipped with an XM10 CCD camera (Olympus, Japan). **(i)** Shown are representative images taken under ×60 magnification. Scale bar = 20µm. **(ii)** Adherent platelets were counted from random visual fields with a total area of $0.1mm^2$. **(iii)** Images of adherent platelets were used to evaluate platelet surface area using ImageJ software (NIH, USA). Data are from 4 independent experiments with separate blood donors and are means ± SD. *: $P<0.05$, **: $P<0.01$.

5.3 Tyrosine phosphorylation occurs in platelets adherent to FXIIIa

The effects observed from Src-family kinase inhibition suggested that protein tyrosine-phosphorylation events were induced in FXIIIa adherent platelets. To examine this, adherent platelets were lysed and inspected for proteins phosphorylated on tyrosine residues using SDS-PAGE and immunoblotting (section 2.8). Platelets were adhered in the presence of apyrase (1U/mL) and indomethacin (10µM) to preclude activation induced by secreted ADP and TXA_2 respectively. Adhesion to FXIIIa resulted in the phosphorylation of multiple proteins with those most prominently phosphorylated appearing at molecular weights of 27, 36, 40, 48, 55, 72 and 130kDa under SDS-PAGE (Figure 5.2A). The profile of tyrosine-phosphorylated proteins matched that from fibrinogen-adherent platelets and in both cases, tyrosine phosphorylation was restricted to resting levels when platelet integrins were blocked with RGDS (Figure 5.2A). These data suggested that FXIIIa triggers integrin-mediated signalling in adherent platelets which is similar to that stimulated by fibrinogen. Signalling induced by FXIIIa was reproducible in platelets adherent on zymogen FXIII (Figure 5.2B), suggesting that transglutaminase activity was not involved in the induction of signalling.

A)


HS
FXIIIa
Fibrinogen
RGDS
-
-
+
-
+
MW (kDa)
220
140
100
80
60
50
40
30
20
IB: pTyr
IB: β-tubulin

B)

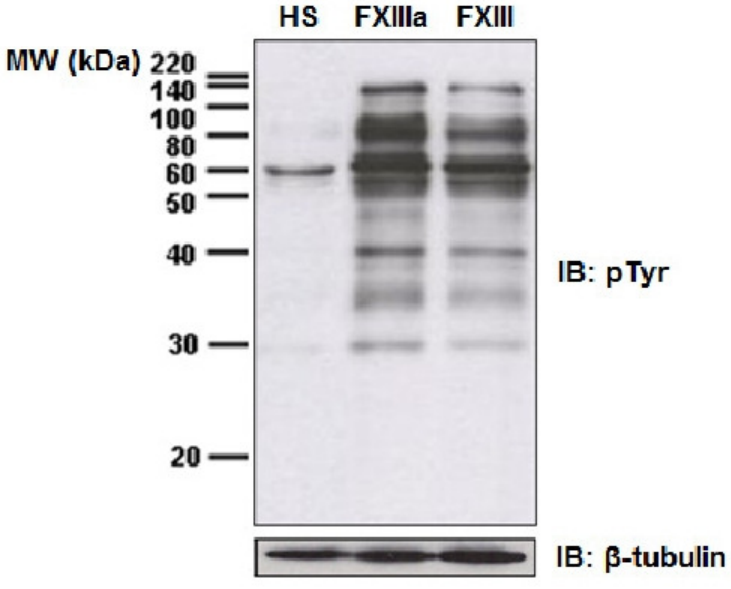

HS
FXIIIa
FXIII
MW (kDa)
220
140
100
80
60
50
40
30
20
IB: pTyr
IB: β-tubulin

Figure 5.2: Adhesion to immobilised FXIII and FXIIIa triggers protein tyrosine-phosphorylation in adherent platelets. **(A)** Wells of a 6-well culture plate were coated overnight at 4°C with activated factor XIII (FXIIIa, 200µg/mL) or heat-inactivated human serum (HS, 5%). Uncoated surfaces were blocked by overlay with HS (5%) for 30min at room temperature. Washed platelets (5×10^8 platelets/mL) were allowed to adhere in the presence of apyrase (1U/mL) and indomethacin (10µM) for 45min at 37°C. In some cases, platelets were pretreated with RGDS (500µM). Adherent-platelet lysates were subjected to SDS-PAGE on a 10-18% gradient gel for 2.5h at 120V under reducing conditions. Separated proteins were then transferred to polyvinylidene fluoride (PVDF) membranes for 2.5h at 100V, immunoblotted (IB) for phosphotyrosine (pTyr) and visualised by enhanced chemiluminescence. Membranes were then stripped and immunoblotted for β-tubulin to assess equal protein loading. The blot is representative of 4 separate experiments with different blood donors. **(B)** As in **(A)** except platelets were also adhered to wells coated with inactive factor XIII (FXIII, 200µg/mL) in the absence of RGDS. MW: molecular weight.

5.4 FXIIIa stimulates platelet signalling through $\alpha_{IIb}\beta_3$, but not $\alpha_v\beta_3$

Since $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ were involved in mediating platelet binding to FXIIIa (section 4.2.2), it was possible that the observed signalling events occurred downstream of either or both receptors. To specifically determine whether each receptor transmitted signals, platelets were treated with either LM609 (20μM) or tirofiban (2μM), adhered to FXIIIa, and then their lysates examined by immunoblotting. Blockade of $\alpha_{IIb}\beta_3$ with tirofiban restricted protein tyrosine-phosphorylation to basal levels which was unrestrained following specific inhibition of $\alpha_v\beta_3$ with LM609 (Figure 5.3). These data were consistent with those from spreading measurements where $\alpha_{IIb}\beta_3$, but not $\alpha_v\beta_3$ mediated platelet spreading (Figure 4.7). Taken together, these data suggest that $\alpha_{IIb}\beta_3$, but not $\alpha_v\beta_3$ triggers activatory platelet signalling which leads to spreading responses in FXIIIa-adherent platelets.

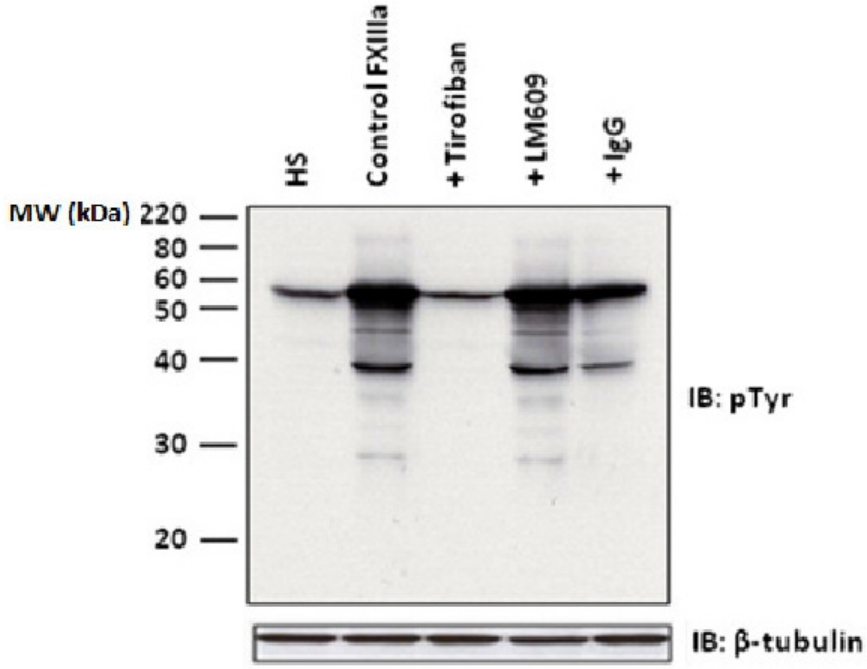


Figure 5.3: FXIIIa stimulates protein tyrosine-phosphorylation through $\alpha_{IIb}\beta_3$, but not $\alpha_v\beta_3$ in adherent platelets. Wells of a 6-well culture plate were coated overnight at 4°C with activated factor XIII (FXIIIa, 200µg/mL) or heat-inactivated human serum (HS, 5%). Uncoated surfaces were blocked by overlay with HS (5%) for 30min at room temperature. Washed platelets (5×10^8 platelets/mL) were allowed to adhere in the presence of apyrase (1U/mL) and indomethacin (10µM) for 45min at 37°C. In some cases, platelets were pretreated with tirofiban (2µM), LM609 (20µg/mL), IgG control antibody (20µg/mL) or modified Tyrode's buffer (control) for 20min at 37°C. Adherent-platelet lysates were subjected to SDS-PAGE on a 10-18% gradient gel for 2.5h at 120V under reducing conditions. Separated proteins were then transferred to polyvinylidene fluoride (PVDF) membranes for 2.5h at 100V, immunoblotted (IB) for phosphotyrosine (pTyr) and visualised by enhanced chemiluminescence. Membranes were then stripped and immunoblotted for β-tubulin to assess equal protein loading. The blot is representative of 3 separate experiments with different blood donors. MW: molecular weight.

5.5 Specific identification of tyrosine-phosphorylated proteins in FXIIIa-adherent platelets

Having established that FXIIIa could stimulate platelet signalling uniquely through $\alpha_{IIb}\beta_3$, some of the intracellular proteins involved were identified. Signalling downstream of $\alpha_{IIb}\beta_3$ is characterised by Src-mediated phosphorylation and activation of Syk which sequentially phosphorylates the adaptor protein SLP-76 and PLCγ2 (Obergfell et al., 2002). Similar events occur downstream of glycoprotein VI (GPVI); another crucial platelet receptor, but are distinguishable by Syk-mediated phosphorylation of the adaptor protein LAT which does not occur downstream of $\alpha_{IIb}\beta_3$ (Watson et al., 2005). Thus, in order to isolate the tyrosine kinase-dependent signalling pathway activated in FXIIIa-adherent platelets, tyrosine-phosphorylation of Syk, SLP-76, PLCγ2 and LAT were examined using immunoprecipitation and western blotting techniques.

5.5.1 Optimisation of protein-immunoprecipitation from adherent platelets

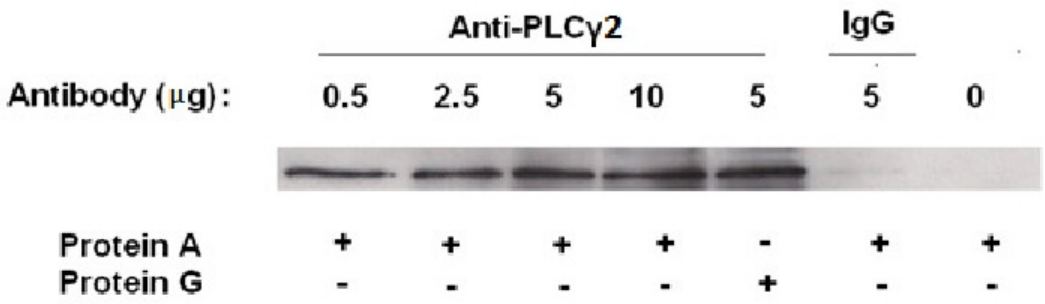
In order to efficiently immunoprecipitate each protein, the technique required optimisation with respect to quantity of antibody and type of beads used as a solid-phase for antibody-binding. Increasing amounts of antibodies specific for each target protein were bound to fixed amounts of either protein-A or protein-G beads. Antibody-conjugated beads were then incubated with unstimulated platelet-lysates to establish the amount of each antibody that could immunoprecipitate detectable protein levels most economically. Immunoprecipitated proteins were then subjected to SDS-PAGE, transferred to PVDF membranes and identified by immunoblotting (section 2.8.2.1).

Data obtained using an anti-PLCγ2 antibody revealed that PLCγ2 could be detected by 0.5µg of antibody and increasing the amount of antibody beyond 5µg failed to increase the yield of immunoprecipitated PLCγ2 with protein-A beads (Figure 5.4A). Comparable PLCγ2 levels were obtained using protein-G beads with 5µg of antibody, indicating no superior advantage over protein-A beads. Importantly, PLCγ2 was undetectable following incubation of platelet lysates with protein-A beads coated with a control IgG antibody that was non-reactive against PLCγ2, indicating the absence of non-specific binding of PLCγ2 by added antibodies. Furthermore, protein-A beads alone failed to pull down PLCγ2, also indicating that they did not bind it non-specifically.

For immunoprecipitation experiments, a fixed amount of bead-slurry (25µL) was used to bind antibodies. To investigate whether this amount of beads limited the surface available for antibody-conjugation and the resulting protein yield, greater amounts of Protein-A bead-slurry up to 75µL were incubated with 5µg of anti-PLCγ2 antibody. Increasing the amount of slurry over 25µL failed to increase the yield of immunoprecipitated PLCγ2, indicating that it was not limiting (Figure 5.4). From these data, it was concluded that using 5µg of anti-PLCγ2 antibody with 25µL of protein-A beads would suffice for subsequent experiments.

Using a similar approach to that described in detail for PLCγ2, inference from data in Figure 5.5 led to the use of anti-SLP-76 (5µg) and of anti-LAT (5µg) antibodies with protein-G beads. Syk was immunoprecipitated with anti-Syk antibody (5µg) with protein-A beads in accordance with optimisation previously performed in the lab (Riba et al., 2008).

A)



B)

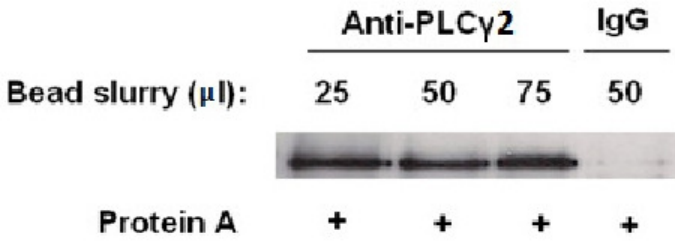
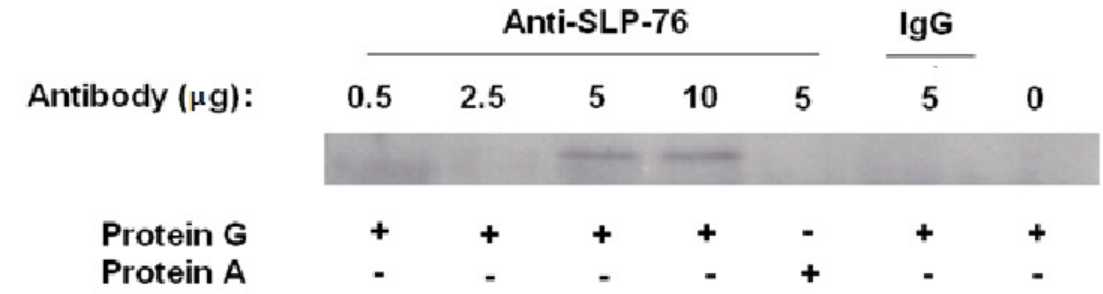


Figure 5.4: Optimisation of amounts of antibody and bead slurry required for PLCγ2 immunoprecipitation. A suspension of either protein-A or protein-G sepharose beads (50%, w/v in TBS-T) was prepared (slurry). **(A)** Anti-PLCγ2 antibody (0.5-10μg) or non-reactive IgG (5μg) were incubated with slurry (25μL) for 1h at 4°C to allow conjugation with beads. Antibody-bead conjugates were then incubated with precleared lysates of unstimulated platelets (5×10^8 platelets/mL) overnight at 4°C to immunoprecipitate PLCγ2. In some cases, unconjugated slurry was incubated with platelet lysates to assess non-specific protein binding. Beads were then pelleted by centrifugation at 8500*g* for 1min, washed sequentially with lysis buffer and TBS then boiled in Laemmli buffer to liberate antibody-PLCγ2 complexes. Immunoprecipitates were separated by SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membranes, immunoblotted for PLCγ2 with anti-PLCγ2 antibody (1:1000) and visualised by enhanced chemiluminescence. **(B)** As in **(A)**, except anti-PLCγ2 antibody (5μg) or non-reactive rabbit IgG (5μg) were incubated with increasing amounts of slurry (25-75μL). Blots are from single experiments performed in duplicate.

A)



B)

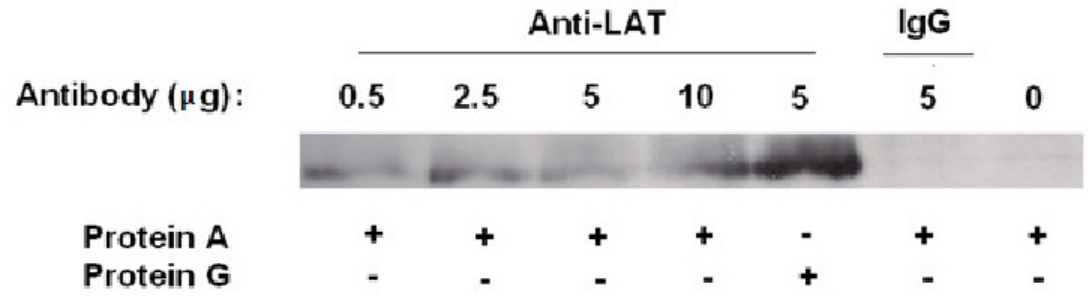


Figure 5.5: Optimisation of amounts of antibody and bead slurry required for SLP-76 and LAT immunoprecipitation. Suspensions of either protein-A or protein-G sepharose beads (50%, w/v in TBS-T) were prepared (slurry). **(A)** Anti-SLP-76 (0.5-10µg) or non-reactive IgG (5µg) antibodies were incubated with slurry (25µL) for 1h at 4°C to allow conjugation with beads. Antibody-bead conjugates were then incubated with precleared lysates of unstimulated platelets (5×10^8 platelets/mL) overnight at 4°C to immunoprecipitate each protein. In some cases, slurry alone was incubated with platelet lysates to assess non-specific protein binding. Beads were then pelleted by centrifugation at 8500*g* for 1min, washed sequentially with lysis buffer and TBS then boiled in Laemmli buffer to liberate antibody-antigen complexes. Immunoprecipitates were separated by SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membranes then immunoblotted for SLP-76 using anti-SLP-76 (1:1000) and visualised by enhanced chemiluminescence. **(B)** As in **(A)**, except lysates were immunoprecipitated for LAT by conjugating increasing amounts of anti-LAT antibody (0.5-10µg) to bead slurry (25µL) and membranes immunoblotted with anti-LAT (1:1000). Blots are from single experiments performed in duplicate.

5.5.2 Syk, PLCγ2, SLP-76, but not LAT are tyrosine-phosphorylated in FXIIIa-adherent platelets.

Having optimised the immunoprecipitation technique, it was applied to FXIIIa-adherent platelets. Each of the targeted proteins was immunoprecipitated sequentially from the same lysates to ensure that all phosphorylation events occurred in the same platelets. Immunoprecipitates were subjected to SDS-PAGE, transferred to PVDF membranes and immunoblotted initially with an anti-phosphotyrosine antibody then with antibodies against each target protein to ensure equal loading in each lane of the gel.

Syk (72kDa molecular weight) was tyrosine-phosphorylated in FXIIIa and fibrinogen-adherent platelets, but not those suspended over human serum (Figure 5.6A). Importantly, the IgG control antibody failed to immunoprecipitate Syk, indicating the absence of non-specific antibody binding. Immunoblotting with an anti-Syk antibody showed that equal amounts of immunoprecipitated Syk were present in each lane. Similarly, PLCγ2 (155kDa) (Figure 5.6B) and SLP-76 (76kDa) (Figure 5.6C) were tyrosine-phosphorylated in FXIIIa and fibrinogen-adherent platelets indicating their involvement in tyrosine kinase-mediated signalling. Also observable in the blots were the heavy chains (55kDa) and light chains (25kDa) of antibodies used to immunoprecipitate each protein (Figure 5.6).

To isolate the tyrosine-kinase signalling pathway occurring in FXIIIa-adherent platelets, LAT was immunoprecipitated and its tyrosine-phosphorylation assessed. LAT remained unphosphorylated in both FXIIIa and fibrinogen-adherent platelets (Figure 5.7A). In contrast,

its tyrosine-phosphorylation was stimulated by collagen (Figure 5.7B). Taken together with data in Figure 5.3, these data suggest that FXIIIa stimulates tyrosine kinase-mediated signalling through $\alpha_{IIb}\beta_3$ which is similar to that stimulated by fibrinogen, but not collagen.

5.6 FXIIIa does not stimulate tyrosine-phosphorylation in suspended platelets

FXIII circulates in plasma where it can also bind suspended platelets (Devine et al., 1993, Cox and Devine, 1994). Having established that FXIII stimulated signal-transduction in adherent platelets when immobilised, its ability to do the same in suspension was also investigated. Washed platelets (5×10^8 platelets/mL) were incubated with FXIIIa for up to 60min under constant stirring conditions. Manganese ions (1mM) were added to artificially give integrins high affinity conformations without stimulating "inside-out" signalling (Mould et al., 2002, Chen et al., 2003). Platelets were subsequently lysed and the lysates separated by SDS-PAGE then immunoblotted with an anti-phosphotyrosine antibody. In contrast to platelets that had been incubated with collagen, no significant increase in tyrosine-phosphorylation was observed with suspended FXIIIa (Figure 5.8).

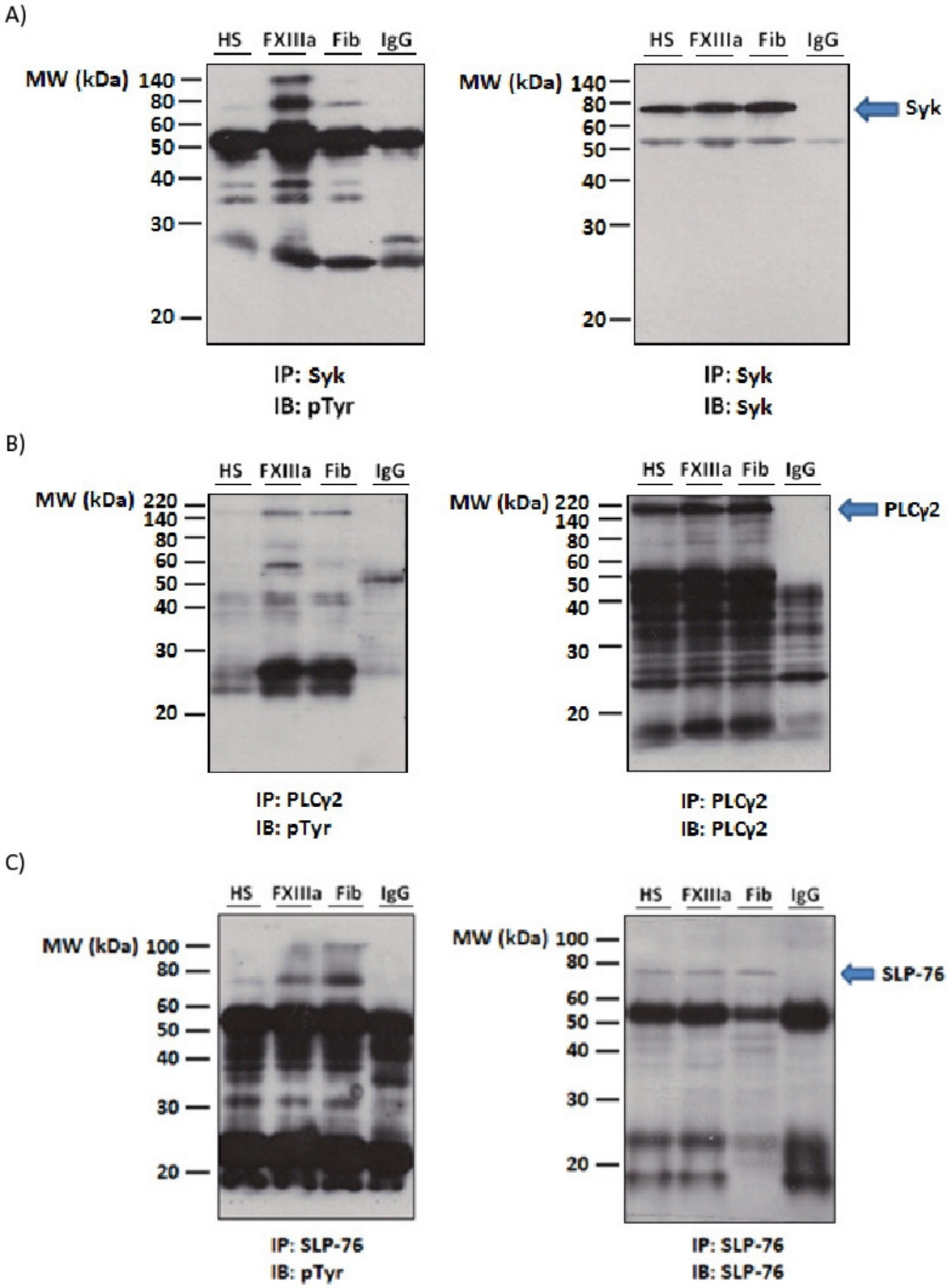
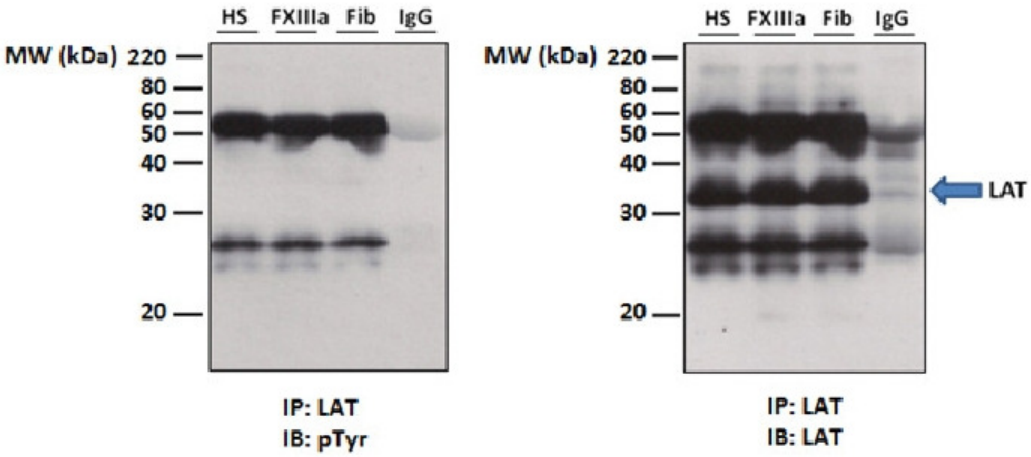

A)
HS FXIIIa Fib IgG
MW (kDa) 140 80 60 50 40 30 20
IP: Syk
IB: pTyr
HS FXIIIa Fib IgG
MW (kDa) 140 80 60 50 40 30 20
Syk
IP: Syk
IB: Syk
B)
HS FXIIIa Fib IgG
MW (kDa) 220 140 80 60 50 40 30 20
IP: PLCγ2
IB: pTyr
HS FXIIIa Fib IgG
MW (kDa) 220 140 80 60 50 40 30 20
PLCγ2
IP: PLCγ2
IB: PLCγ2
C)
HS FXIIIa Fib IgG
MW (kDa) 100 80 60 50 40 30 20
IP: SLP-76
IB: pTyr
HS FXIIIa Fib IgG
MW (kDa) 100 80 60 50 40 30 20
SLP-76
IP: SLP-76
IB: SLP-76

Figure 5.6: Syk, PLCγ2 and SLP-76 are tyrosine-phosphorylated in platelets adherent on FXIIIa. Wells of a 6-well culture plate were coated overnight at 4°C with heat-inactivated human serum (HS, 5%), activated factor XIII (FXIIIa, 200µg/mL) or fibrinogen (Fib, 200µg/mL). Uncoated surfaces were blocked by overlay with HS (5%) for 30min at room temperature. Washed platelets (5×10^8 platelets/mL) were allowed to adhere in the presence of apyrase (1U/mL) and indomethacin (10µM) for 45min at 37°C. Syk: **(A)**, PLCγ2: **(B)** and SLP-76: **(C)** were sequentially immunoprecipitated from adherent platelet lysates using specific antibodies (all 5µg with 25µL bead slurry) overnight at 4°C. Protein-A beads were used for Syk and PLCγ2 while protein-G beads were used for SLP-76. Immunoprecipitation with non-reactive antibody (IgG, 5µg) was a control against non-specific antibody interactions. Immunoprecipitates were subjected to SDS-PAGE on a 10-18% gradient gel for 2.5h at 120V under reducing conditions. Separated proteins were then transferred to polyvinylidene fluoride (PVDF) membranes for 2.5h at 100V, immunoblotted against the indicated proteins using specific antibodies (all 1:1000) and visualised by enhanced chemiluminescence. Blots represent 3 independent experiments with platelets from separate blood donors. IB: immunoblotting, MW: molecular weight, pTyr: phosphotyrosine.

A)


HS
FXIIIa
Fib
IgG
MW (kDa)
220
80
60
50
40
30
20
IP: LAT
IB: pTyr
HS
FXIIIa
Fib
IgG
MW (kDa)
220
80
60
50
40
30
20
LAT
IP: LAT
IB: LAT

B)

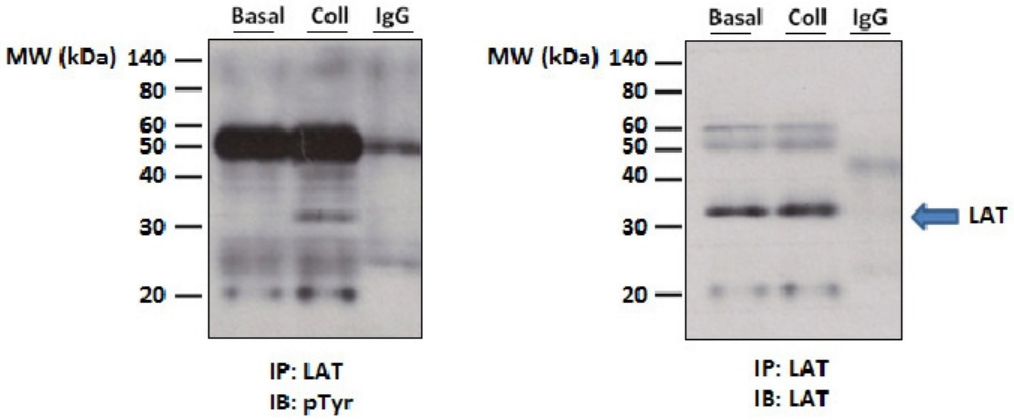
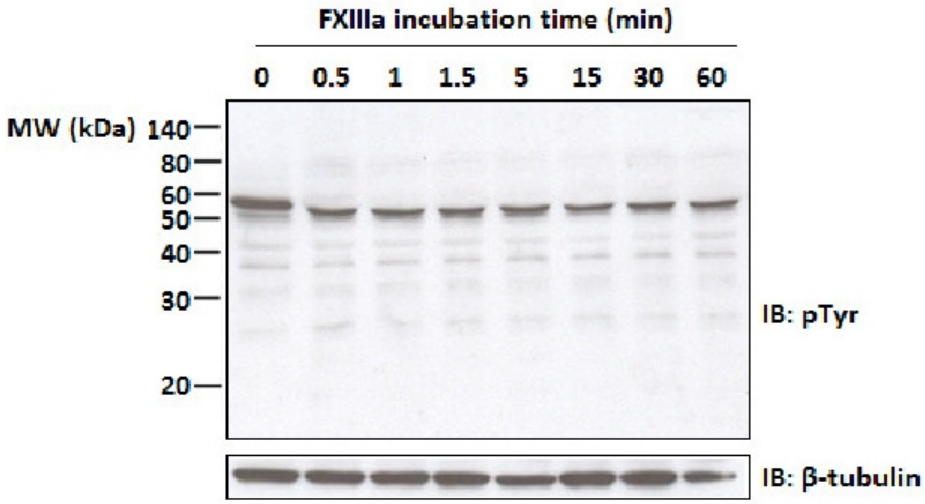

Basal
Coll
IgG
MW (kDa)
140
80
60
50
40
30
20
IP: LAT
IB: pTyr
Basal
Coll
IgG
MW (kDa)
140
80
60
50
40
30
20
LAT
IP: LAT
IB: LAT

Figure 5.7: LAT is not tyrosine-phosphorylated in FXIIIa-adherent platelets. **(A)** Wells of a 6-well culture plate were coated overnight at 4°C with heat-inactivated human serum (HS, 5%), activated factor XIII (FXIIIa, 200µg/mL) or fibrinogen (Fib, 200µg/mL). Uncoated surfaces were blocked by overlay with HS (5%) for 30min at room temperature. Washed platelets (5×10^8 platelets/mL) were allowed to adhere in the presence of apyrase (1U/mL) and indomethacin (10µM) for 45min at 37°C. LAT was immunoprecipitated from adherent platelet lysates using anti-LAT antibody (5µg with 25µL protein-G bead slurry) overnight at 4°C. Immunoprecipitation with non-reactive antibody (IgG, 5µg) was a control against non-specific antibody interactions. Immunoprecipitates were subjected to SDS-PAGE on a 10-18% gradient gel for 2.5h at 120V under reducing conditions. Separated proteins were then transferred to polyvinylidene fluoride (PVDF) membranes for 2.5h at 100V, immunoblotted against the indicated proteins using specific antibodies (all 1:1000) and visualised by enhanced chemiluminescence. **(B)** As in **(A)**, except platelets were adhered to collagen (10µg/mL) in the presence of EGTA (1mM), apyrase (1U/mL) and indomethacin (10µmol/L). Blots are representative of 3 independent experiments with platelets from separate donors. IB: immunoblotting, MW: molecular weight. pTyr: phosphotyrosine.

A)


FXIIIa incubation time (min)
0 0.5 1 1.5 5 15 30 60
MW (kDa) 140
80
60
50
40
30
20
IB: pTyr
IB: β-tubulin

B)

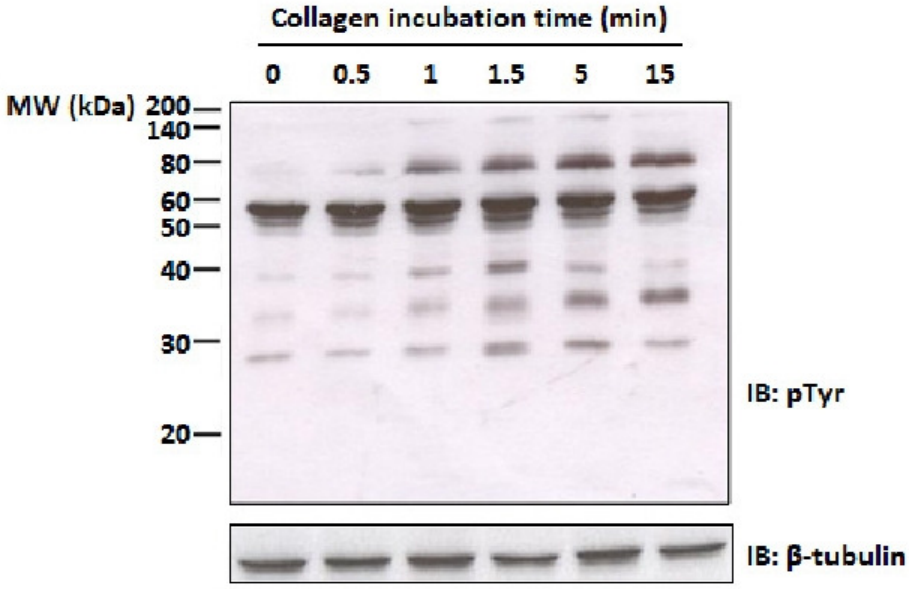

Collagen incubation time (min)
0 0.5 1 1.5 5 15
MW (kDa) 200
140
80
60
50
40
30
20
IB: pTyr
IB: β-tubulin

Figure 5.8: FXIIIa does not stimulate protein tyrosine phosphorylation in suspended platelets. (A) Washed platelets (5×10^8 platelets/mL) were incubated with activated factor XIII (FXIIIa, 200µg/mL) in the presence of manganese ions (1mM), apyrase (1U/mL) and indomethacin (10µM) for various times (0.5-60min) under constant stirring (1000rpm) at 37°C. Platelets were lysed and subjected to SDS-PAGE on a 10-18% gradient gel for 2.5h at 120V under reducing conditions. Separated proteins were then transferred to polyvinylidene fluoride (PVDF) membranes for 2.5h at 100V, immunoblotted against the indicated proteins using specific antibodies (all 1:1000) and visualised by enhanced chemiluminescence. **(B)** As in **(A)**, except suspended platelets were incubated with collagen (50µg/mL) for various durations (0.5-15min) in the presence of EGTA (1mM), apyrase (1U/mL) and indomethacin (10µM). Blots are representative of 3 independent experiments with platelets from separate donors. IB: immunoblotting, MW: molecular weight. pTyr: phosphotyrosine.

5.7 Stable platelet adhesion and spreading on FXIIIa are dependent on intracellular calcium mobilisation.

Signalling downstream of $\alpha_{IIb}\beta_3$ causes activation of PLCγ2 which leads to the mobilisation of intracellular calcium stores and platelet activation (Wonerow et al., 2003). Since PLCγ2 was tyrosine-phosphorylated in FXIIIa-adherent platelets (Figure 5.6), which is associated with its activation (Daniel et al., 1994), the role of intracellular calcium elevation in platelet adhesion to FXIIIa was investigated. Preincubating platelets with BAPTA-AM (20μM), an intracellular calcium chelator (Harrison and Bers, 1987), significantly diminished platelet adhesion to FXIIIa (762±40 to 123±26 platelets/0.1mm^2 for the absence and presence of BAPTA-AM respectively, P<0.01). Moreover, U73122 (5μM), a PLC antagonist (Bleasdale et al., 1990), ablated stable platelet adhesion (5±3 platelets/0.1mm^2 P<0.01 vs control) (Figure 5.9). Together, these data suggest a crucial role for calcium mobilisation in stabilising platelet adhesion to FXIIIa.

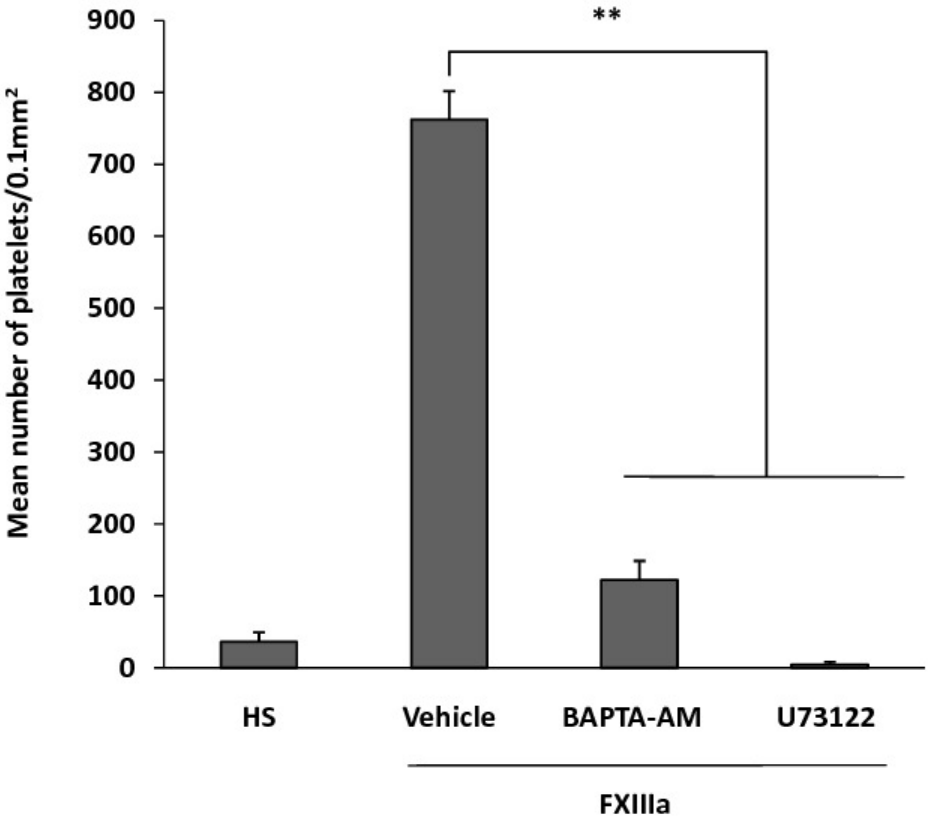


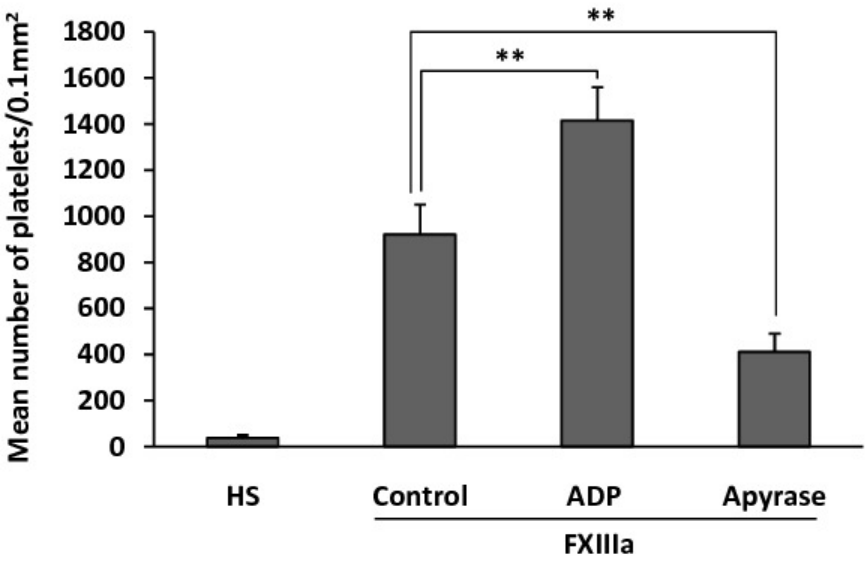
Figure 5.9: Intracellular Ca^{2+} mobilisation is required for stable platelet adhesion on FXIIIa.
Wells on glass slides were coated overnight with heat-inactivated human serum (HS, 5%) or activated factor XIII (FXIIIa, 200µg/mL). Uncoated surfaces were blocked by overlay with HS (5%) for 30min at room temperature. Washed platelets (5×10^7 platelets/mL) were treated with 1,2-bis-(o-aminophenoxy)ethane-tetra-acetic acid tetra-(acetoxymethyl)ester (BAPTA-AM, 20µM), U73122 or 0.01% DMSO (vehicle control) for 20 min at 37°C then adhered to the indicated surface for 1h at 37°C. Adherent platelets were stained with TRITC-phalloidin and viewed under the ×60 objective of an IX71 fluorescence microscope (Olympus, Japan). Platelets from random visual fields with a total area of $0.1mm^2$ were counted. Data shown are means ± SD from 4 independent experiments with separate blood donors. **: $P<0.01$.

5.8 Platelet adhesion to FXIIIa is influenced by platelet activation

Following stimulation by agonists, platelet integrin receptors acquire high affinity conformations and bind more readily to their ligands (Shattil et al., 1998, Shattil and Newman, 2004). To establish the role of agonist-stimulated platelet activation in platelet adhesion to FXIIIa, platelets were preincubated with ADP (1μM) which significantly increased numbers of adherent platelets from 922±128 to 1415±145 platelets/0.1mm^2 (P<0.01) (Figure 5.10A). Since FXIIIa-adherent platelets were observed to secrete their granular contents (section 3.9), the role of secreted ADP was also investigated. Addition of apyrase (1U/mL), an enzyme that decomposes ambient ADP (Pilla et al., 1996), reduced adherent platelet numbers from 922±128 to 412±78 platelets/0.1mm^2 (P<0.01).

In contrast to adhesion, ADP did not influence platelet spreading since it did not change from control values of 29.0±4.0 to 28.7±2.2μm^2 in the presence of ADP and 28.0±3.1 in the presence of apyrase (P>0.05, ANOVA) (Figure 5.10B). These data suggest that although adhesion to FXIIIa is enhanced by platelet activation, integrin binding to FXIIIa provides adequate stimulus to drive platelet spreading.

A)


Mean number of platelets/0.1mm²
1800
1600
1400
1200
1000
800
600
400
200
0
**
**
HS
Control
ADP
Apyrase
FXIIIa

B)

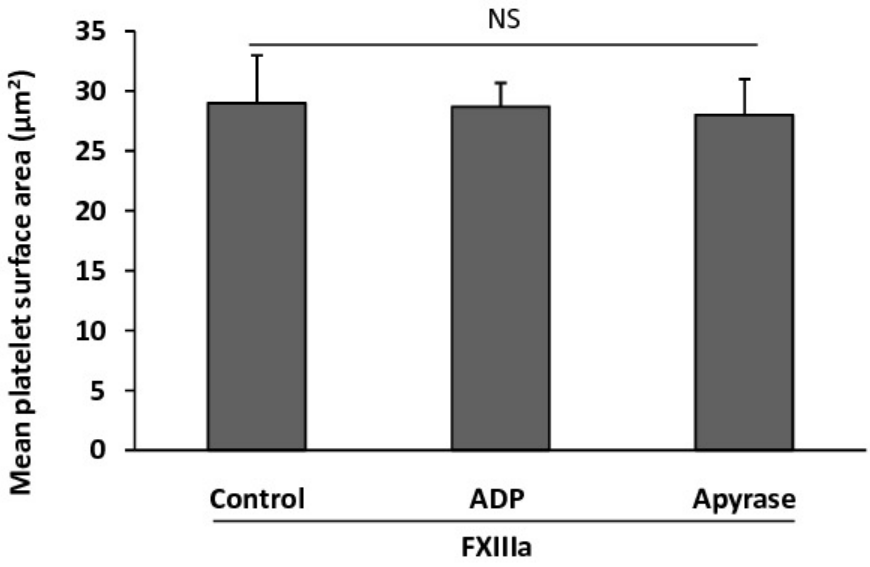

Mean platelet surface area (µm²)
35
30
25
20
15
10
5
0
NS
Control
ADP
Apyrase
FXIIIa

Figure 5.10: ADP-stimulated platelet activation increases adhesion, but not spreading on FXIIIa. Wells on glass slides were coated overnight with heat-inactivated human serum (HS, 5%) or activated factor XIII (FXIIIa, 200µg/mL). Uncoated surfaces were blocked by overlay with HS (5%) for 30min at room temperature. Washed platelets (5×10^7 platelets/mL) were treated with adenosine diphosphate (ADP, 1µM), apyrase (1U/mL) or modified Tyrode's buffer (control) for 20min at 37°C then adhered for 1h at 37°C. Adherent platelets were stained with TRITC-phalloidin and viewed under the ×60 objective of an IX71 fluorescence microscope (Olympus, Japan). **(A)** Platelets from random visual fields with a total area of $0.1mm^2$ were counted. **(B)** Images of adherent platelets were used to evaluate platelet surface area using ImageJ software (NIH, USA). Data shown are means ± SD from 4 independent experiments with separate blood donors. **: $P<0.01$, NS: not significant.

5.9 Platelet inhibition regulates adhesion to FXIIIa

Vascular endothelial cells release NO to restrict circulating platelets to their quiescent states (Furchgott and Zawadzki, 1980, Palmer et al., 1988). To investigate the effects of platelet inhibition by NO on their ability to bind FXIIIa, platelets were preincubated with GSNO, an NO donor (Radomski et al., 1992), prior to adhesion to FXIIIa. Treating platelets with GSNO (10μM) significantly diminished their ability to bind FXIIIa (922±128 to 187±78 platelets/0.1mm^2 for the absence and presence of GSNO, P<0.01) (Figure 5.11).

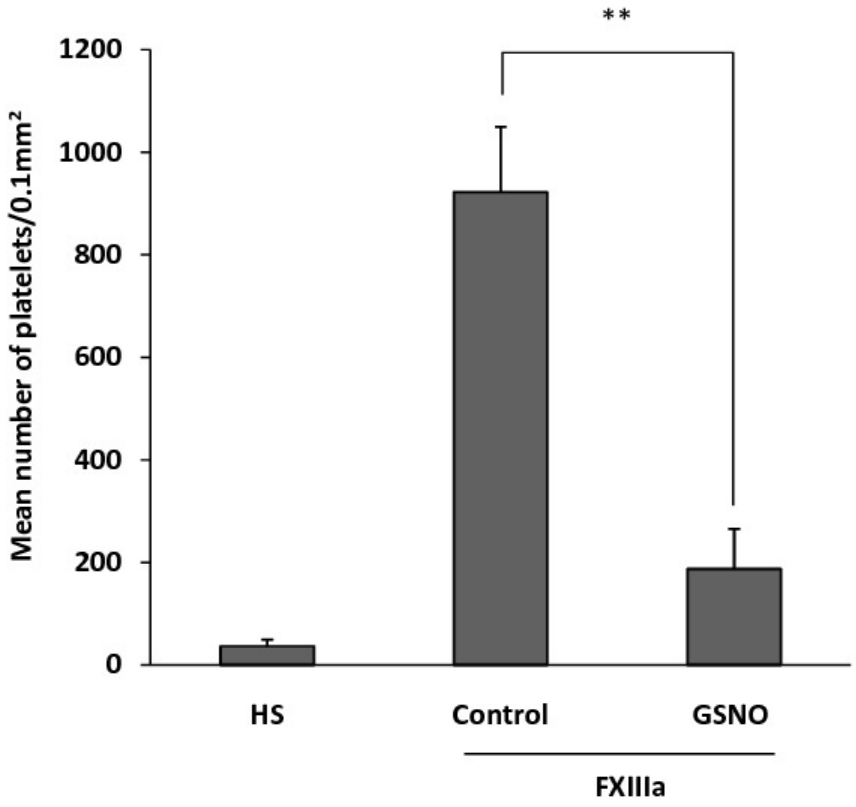


Figure 5.11: Platelet inhibition by nitric oxide diminishes FXIIIa-mediated platelet adhesion.

Wells on glass slides were coated overnight with heat-inactivated human serum (HS, 5%) or activated factor XIII (FXIIIa, 200µg/mL). Uncoated surfaces were blocked by overlay with HS (5%) for 30min at room temperature. Washed platelets (5×10^7 platelets/mL) were treated with GSNO (10µM) or modified Tyrode's buffer (control) for 20min at 37°C then adhered for 1h at 37°C. Adherent platelets were stained with TRITC-phalloidin and viewed under the ×60 objective of an IX71 fluorescence microscope (Olympus, Japan). Platelets from random visual fields with a total area of $0.1mm^2$ were counted. Data shown are means ± SD from 4 independent experiments with separate blood donors. **: $P<0.01$.

5.10 Discussion

Biochemical signalling is central to the induction of activatory responses which accompany platelet adhesion to thrombogenic ligands. It is this activatory signalling which stimulates platelet shape changes, granular secretion, phosphatidylserine exposure, stable adhesion and aggregation (Ruggeri, 2002, Varga-Szabo et al., 2008, Rivera et al., 2009). The present study demonstrates for the first time that FXIIIa is able to initiate signalling in platelets thereby stimulating characteristic functional responses. In particular, FXIIIa induced signalling through $\alpha_{IIb}\beta_3$ integrins which led to spreading of adherent platelets (sections 4.3 and 5.4). This relied on the activity of Src-family kinases, since spreading and its dependent stable adhesion were blocked following their inhibition (Figure 5.1). Consistent with the activation of tyrosine-kinases, adhesion to FXIIIa caused tyrosine phosphorylation of key platelet signalling proteins including Syk, SLP-76 and PLCγ2 independently of platelet-derived agonists ADP and TxA_2 (Figure 5.6). Tyrosine phosphorylation was also stimulated in platelets adherent to zymogen FXIII suggesting that it was independent of transglutaminase activity (Figure 5.2B). Thus, the signalling events initiated by FXIIIa resemble those in platelets adherent on fibrinogen, which also signals through $\alpha_{IIb}\beta_3$ (Gao et al., 1997, Obergfell et al., 2001, Shattil, 2005). Although the same secondary messengers appear to be involved in signalling that follows adhesion to FXIIIa and fibrinogen, there are observable differences in the extents of their phosphorylation in platelets that have adhered for 45min. For instance, Syk appears to be more heavily phosphorylated in FXIIIa-adherent platelets than in those adherent to fibrinogen, while the converse is true for SLP-76 (Figure 5.6). This suggests that signalling downstream of FXIIIa and fibrinogen may follow different kinetics. Since, platelet-

associated FXIIIa cross-links several ligands which occupy integrins (Dale et al., 2002), the ensuing signalling may explain the differences observed. Nevertheless, fuller appreciation of the nature of phosphorylation of each secondary messenger would be enabled by conducting time-course experiments with platelets that would have been allowed to adhere for times other than 45min.

"Outside-in" signalling downstream of $\alpha_{IIb}\beta_3$ is characterised by activation of Src-family kinases which leads to the recruitment of the tyrosine kinase Syk (Gao et al., 1997, Obergfell et al., 2002). Sequentially, PLCγ2 is activated, leading to Ca^{2+} mobilisation required for platelet activation, causing further $\alpha_{IIb}\beta_3$ high affinity conformational changes ("inside-out signalling"). Hence, integrin activation is self-propagating and influenced by platelet activation. Indeed, platelet adhesion to FXIIIa was enhanced by ADP-stimulated platelet activation (Figure 5.10A). In line with this, platelet adhesion to FXIIIa was inhibited by NO (Figure 5.11), a physiological endothelium-derived platelet antagonist. This suggests that adhesion only takes place at regions where platelet inhibition is diminished and platelet activation promoted such as at sites of vascular injury or where the endothelium is dysfunctional.

Platelets have multiple receptors for many of their ligands and some of these receptors mediate differential, non-redundant effects. For example, ADP stimulates platelets through $P2Y_1$ and $P2Y_{12}$ purinergic receptors with the former leading to PKC activation and Ca^{2+} mobilisation while the latter diminishes intracellular cAMP levels (Daniel et al., 1998). Similar multiplicity holds true for vWF, thrombin and collagen receptors (Rivera et al., 2009). In

particular, collagen and vWF-mediate platelet activation through mechanisms which resemble those by FXIIIa which triggers tyrosine kinase-dependent signalling downstream of $\alpha_{IIb}\beta_3$, but not $\alpha_v\beta_3$ (Figure 5.3). Firstly, collagen drives activatory signalling through GPVI while $\alpha_2\beta_1$ appears more limited to an adhesive role (Watson et al., 2005). Secondly, collagen stimulates protein tyrosine-phosphorylation with the involvement of Src, Syk, and SLP-76 leading to activation of PLCγ2 and cellular Ca^{2+} mobilisation (Watson and Gibbins, 1998). Immobilised vWF binds flowing platelets only transiently through GP1b/IX/V and requires association with $\alpha_{IIb}\beta_3$ for more stable attachment to initiate spreading responses (Ruggeri, 2007a). Thus, it is unsurprising that FXIIIa binds platelets through the dual-receptor mechanism described here.

Since $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ share a common β subunit and considerable homology between their α subunits (Shattil and Newman, 2004) signalling by one integrin, but not the other may be unexpected. $\alpha_v\beta_3$ was first discovered in melanoma cells where its ligand affinity for vitronectin, vWF and fibrinogen was established (Cheresh and Spiro, 1987). The receptor was also identified in endothelial cells where it had similar ligand affinity and its blockade inhibited angiogenesis as well as induced apoptosis (Brooks et al., 1994, Brooks et al., 1995). In these cells, $\alpha_v\beta_3$ was reported to stimulate a signalling cascade involving FAK, calpain, NF-κB, and Rac (Shattil, 1995). Signalling mediated by $\alpha_v\beta_3$ has also been well characterised in osteoclasts where it involves activation of Src and Syk through mechanisms similar to those downstream of platelet $\alpha_{IIb}\beta_3$ (Zou et al., 2007). Since $\alpha_v\beta_3$ and both tyrosine kinases are present in platelets, the existence of a similar signalling cascade would be tempting to speculate. Nevertheless, although platelet studies have demonstrated that $\alpha_v\beta_3$ can mediate

adhesion to vitronectin, fibrinogen and thrombospondin, even by platelets from patients with GT (Lam et al., 1989, Lawler and Hynes, 1989, Coller et al., 1991), there is limited evidence regarding its signalling properties. However, McCarty et al., (2004) reported that adhesion of platelets from $\alpha_{IIb}^{-/-}$ deficient mice to fibrinogen could be inhibited by blocking $\alpha_v\beta_3$. Importantly, although these platelets could adhere to fibrinogen, they failed to spread suggesting that only $\alpha_{IIb}\beta_3$, but not $\alpha_v\beta_3$ is able to trigger signalling in platelets. Similar results were obtained with fibronectin, suggesting that platelet $\alpha_5\beta_1$ may be involved in adhesion, but not signalling and spreading on the protein (McCarty et al., 2004). These data suggest that signalling downstream of $\alpha_v\beta_3$ may be a phenomenon operating in other cells, but not platelets. A possible explanation for this may be the low copy number of $\alpha_v\beta_3$ on platelets which might restrict its cross-linking, homotypic oligomerisation and formation of focal adhesions which facilitate integrin-mediated "outside-in" signalling.

Although immobilised FXIIIa stimulated platelet spreading, it is noteworthy that it did not induce shape changes or aggregation (Figure 3.4), or tyrosine phosphorylation in platelet suspensions (Figure 5.8A). This explains in part why previous studies have not reported signalling events induced by this enzyme. Furthermore, signal transduction induced uniquely by immobilised FXIIIa suggests a distinct mechanism that allows it to promote platelet activation only when localised at sites of vascular damage. Since they bind to collagen exposed at these sites, FXIIIa and vWF may further enhance its thrombogenic potential by supplementing activatory signal transduction through a long-lived, integrin-mediated "outside-in" signalling cascade which maintains platelet contacts within aggregates and stimulates clot-retraction (Ruggeri, 2002). Moreover, FXIIIa becomes localised on the

surfaces of dysfunctional endothelium where it is able to recruit platelets (Dardik et al., 2002b). Such abnormal deposition and activation of platelets may have pathological consequences including the aggravation of peripheral vascular disease which is characterised by elevated FXIIIa levels (Shemirani et al., 2008), activated platelets (Matsagas et al., 2002) and dysfunctional endothelium (Esper et al., 2006).

In conclusion, this chapter has described an activatory signalling cascade that occurs in FXIII-adherent platelets which is stimulated specifically through $\alpha_{IIb}\beta_3$ integrins. Such signalling is crucial for the occurrence of spreading responses that stabilise platelet adhesive contacts with the enzyme. Furthermore, it potentially supplements the activation of platelets at sites of vascular injury.

CHAPTER 6 - GENERAL DISCUSSION

The present project aimed to investigate platelet adhesive contacts with FXIII. Previous demonstration of specific FXIII-binding to platelet $\alpha_{IIb}\beta_3$ fuelled speculation that it may have roles in platelet function (Cox and Devine, 1994, Muszbek et al., 1999). However the physiological consequences of platelet-FXIII interactions have remained elusive. This is largely due to previous studies focussing mainly on platelet-FXIII interactions occurring in suspension. While platelets are able to bind suspended FXIII, the importance of these associations with respect to platelet function was difficult to delineate since suspended platelets from FXIII-deficient patients had been shown to aggregate normally (Ozsoylu and Hicsonmez, 1976). However, a more recent study using platelets from three FXIII-deficient patients gave conflicting results since it reported diminished aggregation responses to collagen, ADP and epinephrine (Anwar et al., 2002). Platelet-bound FXIII was also found to retard fibrinolysis and stabilise thrombi by cross-linking α2-antiplasmin specifically to platelet-rich thrombi (Reed et al., 1991). Together with a demonstration that exogenous FXIII activity imparts a procoagulant platelet phenotype (Dale et al., 2002), these findings renewed research interest into the *in vivo* implications of platelet-FXIII associations.

Dardik et al., (2002b) showed that FXIII-coated plastic surfaces supported platelet adhesion. Theirs was the pioneering investigation of platelet interactions with immobilised FXIII. They also reported that *in vivo* plasma supplementation of FXIII-deficient patients with FXIII concentrate enhanced platelet accrual on activated, dysfunctional endothelium *ex vivo*. This

reinforced hypothesised pathophysiological consequences from a study that reported increased platelet-FXIII associations in patients with PVD, but not matched healthy controls (Devine et al., 1993). However, Dardik et al., (2002b) did not demonstrate the purity of their source of FXIII from contaminating platelet adhesive ligands nor did they assess ensuing functional responses by adherent platelets. Whether FXIII also influences platelet recruitment by ECM proteins which are perhaps more involved in platelet thrombosis has since not been investigated.

Using established platelet imaging and biochemical analyses, functional platelet responses stimulated by interactions with FXIII were examined in the present study. Extensive effort was made to ensure that FXIII actually binds platelets independently of other proteins as described in Chapter 3. This included stringent assessment of purity of the source of plasma FXIII used in this study, the demonstration that platelet adhesion was reproducible on plasma protein-free recombinant FXIII and that it could occur independently of proteins secreted from platelet α-granules. Together, these data provide strong evidence to suggest that immobilised FXIII is indeed a true platelet ligand. Interestingly, while platelet adhesion to FXIII can proceed independently of other proteins, FXIII was observed to enhance platelet accrual by key, thrombogenic platelet-ligands, specifically collagen, fibrinogen and vWF (Chapter 4). Thus like other coagulation proteins which have recently been shown to bind platelets including protein C (White et al., 2008) and factor XI (White-Adams et al., 2009), FXIII may serve to enhance platelet thrombosis under physiological conditions with its abnormally high plasma concentrations predisposing to cardiovascular disease (Devine et al., 1993, Bereczky et al., 2007, Shemirani et al., 2008).

This study presents a potentially new dual-receptor mechanism which facilitates platelet adhesion to FXIII. The mechanism relies on the concerted roles of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins, whereby both integrins are involved in the initial platelet adhesion (Chapter 4) while activatory signal transduction which leads to spreading and stable adhesion is only mediated by $\alpha_{IIb}\beta_3$ (Chapter 5). The involvement of integrins in the adhesion events is paradoxical since FXIII-A lacks both the canonical RGD integrin-binding motif and a KQAGDV sequence (Ichinose and Davie, 1988). The latter is present on fibrinogen γ-chains and crucially mediates physiological fibrinogen-binding by $\alpha_{IIb}\beta_3$ (Kloczewiak et al., 1982, Kloczewiak et al., 1983). However, FXIII-A possesses a leucine-aspartate-valine (LDV) sequence recognised by some integrins (Ichinose and Davie, 1988, Humphries et al., 2006). Since the LDV residues are at opposite ends of FXIII-A_2 dimers in solution (Yee et al., 1995), they are potentially accessible to both integrins.

The model proposed for physiological platelet interactions with FXIII involves multipronged associations. Since much of the FXIII zymogen may circulate bound to fibrinogen (Greenberg and Shuman, 1982, Siebenlist et al., 1996), initial delivery of FXIII to vascular injury sites is likely to occur with it in complex with fibrinogen. At these sites, fibrinogen is captured and immobilises on adherent platelets, where FXIII may enhance fibrinogen-mediated platelet accrual as demonstrated under flow (Figure 4.12). It has been shown that fibrinogen-occupation of $\alpha_{IIb}\beta_3$ does not inhibit platelet-FXIII binding (Cox and Devine, 1994). Furthermore, $\alpha_v\beta_3$ plays a negligible role in platelet fibrinogen-binding (Smith et al., 1990),

but an important role in FXIII-binding (Figure 4.4). Together, these data suggest that platelet binding to FXIII is indeed feasible in the presence of fibrinogen.

Due to the high local thrombin concentrations generated at vascular injury sites, zymogen FXIII is activated and liberated from fibrinogen (Muszbek et al., 1999). It thus becomes free to perform its role as a transglutaminase, cross-linking and stabilising fibrin strands. In addition, FXIIIa acquires the ability to localise on collagen fibres, a property unique from its zymogen form (Figure 4.13). By providing adhesive sites which bind $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$, FXIIIa potentially increases platelet binding sites available per collagen fibre, facilitating increased platelet accrual as evidenced by greater thrombus volumes observable on FXIIIa-bound collagen under flow (Figure 4.14iii). Indeed, localisation of FXIIIa on the ECM is well established and has been shown to be critical for fibroblast proliferation and migration which are compromised causing poor wound healing in FXIII-deficient patients (Muszbek et al., 1999, Wozniak and Noll, 2002). Similar to other integrin ligands, platelet contacts with FXIII are influenced by shear forces imposed by flowing blood. At high shear rates typically found in arterial vessels, vWF is essential to facilitate platelet-FXIIIa contacts which are significantly enhanced by its presence (Figure 4.15). Since vWF rapidly binds to collagen exposed at vascular injury sites (Pareti et al., 1986), it is available to enable platelet interactions with FXIIIa at these sites.

Future work

While the present study has provided new insights into the nature of platelet associations with FXIII a number of issues remain to be considered in future investigations. These include the following:

1. The exact nature and location of the platelet-binding site is yet to be established. It is known that platelets bind to FXIII-A, not FXIII-B (Greenberg and Shuman, 1984), but the exact protein domain interacting with platelets has not been characterised. Definitive demonstration of the involvement of LDV residues or yet other potentially novel integrin-binding sequences within FXIII-A awaits future investigation. This could be tackled in the first instance by genetic manipulation whereby FXIII-A with mutated LDV sequences is examined for its ability to support platelet adhesion. If LDV sequences are shown to be uninvolved, platelet adhesion to peptides fragmented from FXIII-A could be assessed to locate the binding site.

2. The role of $\alpha_v\beta_3$ in platelet adhesion to FXIII as described in this study requires confirmation. Presently, predicted involvement of this integrin derives solely from the use of an inhibitory antibody (LM609). While the use of this antibody to identify potential $\alpha_v\beta_3$ ligands is well-established (Takagi et al., 1997, Jalali et al., 2001), the current observations will be strengthened if they are reproducible with CHO cells transfected with DNA to express $\alpha_v\beta_3$ integrins. Moreover, the use of platelets

specifically deficient in α_v or α_{IIb} will enable confirmation of the dual-receptor mechanism hypothesised to mediate platelet-FXIII contacts.

3. The study of platelet-FXIII interactions in living animals has not been performed to date. Although the present study has aided appreciation of the influence of vascular flow conditions on the stringency of such interactions, their analysis in a more complex *in vivo* setting would provide the clearest indication. FXIII-deficient mice are available and have extended bleeding times (Lauer et al., 2002). Using these mice, platelet thrombus formation can be studied in real-time with *in vivo* models based on intravital microscopy. By fluorescently tagging FXIII, its actual immobilisation and platelet-interaction at vascular injury sites can be assessed. More information can also be obtained by generating $FXIII^{-/-}/\alpha_{IIb}\beta_3^{-/-}$ and $FXIII^{-/-}/\alpha_v\beta_3^{-/-}$ double-knockout mice.

4. The roles of platelet-FXIII interactions in vascular pathology still await specific intensive investigation. The mechanisms involved in FXIII-mediated enhancement of thrombosis shown in this study may be potentially involved in the aggravation of cardiovascular disease. In the future, investigations of platelet thrombus formation leading to vascular occlusion in animals genetically manipulated to express elevated plasma FXIII levels will clarify the involvement of these events in disease. Furthermore, analysis of platelet-FXIII interactions in animals reared on a high-fat diet will provide understanding of their roles in the pathogenesis of atherosclerosis.

Conclusion

Data presented in this thesis contribute to the better understanding of potential *in vivo* consequences of platelet interactions with FXIII which has been demonstrated to induce a number of platelet functional effects such as signal transduction, spreading, secretion and thrombus formation under flow, specifically when immobilised. In particular, FXIII-mediated enhancement of platelet accrual by key platelet ligands which appears to be influenced by FXIII concentration provides a potential premise for reported involvement of high plasma FXIII antigen levels in thrombotic disease.

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ORIGINAL ARTICLE

Factor XIII supports platelet activation and enhances thrombus formation by matrix proteins under flow conditions

S. G. MAGWENZI,* R. A. AJJAN,† K. F. STANDEVEN,† L. A. PARAPIA‡ and K. M. NASEEM*

Centre for Cardiovascular and Metabolic Research, Hull York Medical School, University of Hull, Castle Hill Hospital, Castle Road, Cottingham; †Leeds Institute for Genetics, Health and Therapeutics, University of Leeds, Leeds; and ‡Centre for Atherothrombosis Research, University of Bradford, Bradford, UK

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Summary. *Background:* Activated coagulation factor XIII (FXIIIa) is a transglutaminase that crosslinks fibrin at sites of vascular injury. FXIIIa also associates with blood platelets, although its role in platelet function is unclear and requires clarification. *Objectives:* To evaluate the ability of FXIIIa to support platelet adhesion and spreading under conditions of physiologic flow, and to identify the underpinning receptors and signaling events. *Methods and Results:* Platelet adhesion to immobilized FXIIIa was measured by fluorescence microscopy, and signaling events were characterized by immunoblotting. Immobilized FXIIIa supported platelet adhesion and spreading under static conditions through mechanisms that were dually and differentially dependent on integrins $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$. Platelet adhesion was independent of FXIIIa transglutaminase or protein disulfide isomerase activity. Moreover, adhesion was abolished by antibodies that prevented interaction with FXIIIa, but maintained when potential interactions with fibrinogen were blocked. Platelet adhesion to FXIIIa was reduced significantly by either the specific $\alpha_{IIb}\beta_3$ antagonist tirofiban or the selective $\alpha_v\beta_3$-blocking antibody LM609, and abolished when they were used in combination. Importantly, platelet adhesion was preserved under venous and arterial flow conditions in which both integrins played essential roles. In contrast, FXIIIa stimulated the formation of filopodia and lamellipodia in adherent platelets that was mediated exclusively by $\alpha_{IIb}\beta_3$ and eliminated by the Src-family inhibitor 4-amino-5-(4-methylphenyl-7-(t-butyl)pyrazolo(3,4-d)pyrimidine, indicating a tyrosine kinase-dependent mechanism. Crucially, under conditions of arterial shear, FXIIIa accentuated platelet recruitment by von Willebrand factor and collagen. *Conclusions:* Our data demonstrate a potential role for FXIIIa in supporting platelet adhesion at sites of vascular damage, particularly in association with other thrombogenic matrix proteins.

Correspondence: Khalid M. Naseem, Daisy Building Research Laboratories, Hull York Medical School, University of Hull, Castle Hill Hospital, Castle Road, Cottingham, UK.
Tel.: +44 1482 461905; fax: +44 1482 461848.
E-mail: khalid.naseem@hyms.ac.uk

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Introduction

The accrual of blood platelets at sites of vascular injury is an essential hemostatic response that precludes loss of blood. A plethora of studies have examined the involvement of extracellular matrix (ECM) proteins such as collagen and von Willebrand factor (VWF) in platelet–vessel wall interactions, and inherent adhesive mechanisms have become well established [1]. More recently, a number of proteins involved in the blood coagulation cascade, including protein C and factor XI, have been shown to support platelet adhesion under flow, adding a new dimension to our current understanding of the recruitment of platelets to sites of vascular injury [2,3]. It has been known for some time that platelets also interact with FXIII. FXIII is a member of the transglutaminase family of enzymes, which covalently join substrate molecules through ε(γ-glutamine)–lysine crosslinkages [4]. Plasma FXIII circulates as a tetramer of two carrier B-subunits (FXIII-B) and two catalytic A-subunits (FXIII-A). Upon vascular injury, thrombin cleaves 4-kDa activation peptides from each of the A-subunits, allowing their Ca^{2+}-dependent dissociation from B-subunits and engagement with substrate molecules. A major role for activated plasma FXIII (FXIIIa) is fibrin-clot stabilization through crosslinking reactions [4,5]. Furthermore, a small amount of plasma FXIII is absorbed by platelets and localizes in their α-granules [6]. Following platelet activation, α-granule FXIII may be released to supplement its concentration at sites of developing thrombi [6].

Platelets are known to bind FXIIIa through $\alpha_{IIb}\beta_3$ integrins [7]. However, platelets from Glanzmann thrombasthenia patients, which lack functional $\alpha_{IIb}\beta_3$, maintained an ability to bind FXIIIa, suggesting the involvement of other, unknown, receptors [8]. Observations that FXIIIa binds to platelets

through FXIII-A but not FXIII-B [8] generated considerable interest in the involvement of these interactions in hemostasis and thrombosis [4]. However, the physiologic roles of FXIII in platelet function are largely undefined. FXIIIa at the platelet surface was ascribed a role in the development of highly procoagulant 'coated' platelets [9], suggesting that it may accentuate platelet-dependent thrombus formation. Indeed, investigations by Dardik *et al.* [10] revealed that in vivo supplementation of FXIII-deficient patients with FXIII concentrate almost doubled the tendency of platelets to associate with dysfunctional endothelium *ex vivo*. The investigators demonstrated that, by binding platelets via $\alpha_{IIb}\beta_3$, FXIII(a) facilitated their interactions with endothelial cells. It is thus possible that FXIIIa may play a role in the pathogenesis of atherothrombosis, which is characterized by abnormal platelet–endothelial associations. However, whether FXIIIa also enhances platelet adhesion and thrombus formation on ECM proteins remains unknown. Moreover, its ability to initiate intracellular signaling in platelets or induce characteristic platelet responses such as spreading and thrombus formation under arterial shear has not been characterized.

The aims of the present study were to extend previous findings by Cox and Devine [7] and Dardik *et al.* [10], by determining whether immobilized FXIIIa not only supported platelet adhesion, but also stimulated spreading responses, and to elucidate the receptors and signaling mechanisms involved. We demonstrate that immobilized FXIIIa supports platelet adhesion that triggers signal transduction, leading to spreading and stable adhesion under physiologic flow. These events depend on a dual and non-redundant mediation by $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$. Importantly, FXIIIa synergizes with subendothelial matrix proteins, particularly collagen and VWF, to enhance platelet recruitment under conditions of arterial shear.

Materials and methods

Reagents

Rabbit monoclonal anti-phospholipase C (PLC)γ2 antibody and mouse monoclonal anti-Syk were from Santa Cruz (Heidelberg, Germany). Normal mouse IgG, anti-phosphotyrosine, anti-LAT, anti-SLP-76, anti-$\alpha_v\beta_3$ (LM609) and anti-β-tubulin mAbs were from Millipore (Watford, UK). Mouse monoclonal anti-FXIII-A, fluorescein isothiocyanate-conjugated goat anti-mouse antibody and rabbit polyclonal anti-fibrinogen and anti-FXIII-A antibodies were from Abcam Plc (Cambridge, UK). Phe-Pro-Arg-chloromethylketone (PPACK) was from Cambridge Bioscience (Cambridge, UK). H-Arg-Gly-Asp-Ser-OH (RGDS) and 1,2-bis-(*o*-aminophenoxy)ethane-tetra-acetic acid tetra-(acetoxymethyl) ester (BAPTA-AM) were from Calbiochem (Nottingham, UK). 4-Amino-5-(4-methylphenyl-7-(t-butyl)pyrazolo(3,4-d)pyrimidine (PP1) was from BIOMOL International (Exeter, UK). Collagen horm reagent was from Nycomed (Zurich, Switzerland). U73122 and *S*-nitrosoglutathione (GSNO) were from Tocris Bioscience (Bristol, UK). Purified VWF was from LFB (Lyon, France). Heat-inactivated pooled human serum was from Sheffield University Hospital (Sheffield, UK). Tirofiban (Aggrastat) was from Iroko Pharmaceuticals (Philadelphia, PA, USA). pGEX-6P-1 containing the gene sequence for recombinant FXIII-A (rFXIII-A) was a kind gift from R. Ariëns (University of Leeds, Leeds, UK), and was expressed in BL21-Gold DE3 *Escherichia coli* (Stratagene, CA, USA). Human α-thrombin and all other reagents were from Sigma (Poole, UK).

FXIII activity

Fibrogammin P (CLS Behring, Sussex, UK) was used as a purified source of human plasma-derived FXIII. Fibrogammin P and human rFXIII-A were activated with human α-thrombin (10 U mL^{-1}) in the presence of 10 mM $CaCl_2$ for 1 h at 37 °C. PPACK (20 μM) was then added for 10 min to inactivate thrombin [10]. Complete inhibition of thrombin under these conditions was monitored by its diminished ability to stimulate platelet aggregation and loss of reactivity with a chromogenic substrate (not shown). Transglutaminase activity was monitored by measuring crosslinking of biotinylated amines to fibrinogen, as previously described [11]. To inhibit transglutaminase activity, FXIIIa was incubated with the alkylating agent iodoacetamide (3 mM) for 30 min at 20 °C. Protein disulfide isomerase (PDI) activity was inhibited by incubation with bacitracin (1 mM) for 1 h at 37 °C [12].

Platelet isolation

The experiments were approved by the Post-Graduate Medical Institute ethics committee (University of Hull, UK), in accordance with the declaration of Helsinki. Blood was obtained from healthy volunteers who had denied taking any medication for 2 weeks. Washed platelets were prepared with the prostaglandin E_1 method [13]. Autologous red blood cells (RBCs) were pelleted from whole blood by centrifugation at $200 \times g$ for 20 min at 20 °C. After removal of supernatant, they were washed twice with wash buffer (10 mM EDTA, 50 mM glucose, 90 mM NaCl, 36 mM citric acid, 50 mM KCl, pH 6.5), and then once with modified Tyrode's buffer [13] by further centrifugation at $800 \times g$ for 12 min at 20 °C. When required, they were reconstituted to 50% (v/v) with washed platelets (2×10^8 platelets mL^{-1}, final concentration) in modified Tyrode's buffer supplemented with 1 mM $CaCl_2$ and 40 μM PPACK.

Fluorescence microscopy

Wells on glass slides were coated overnight with heat-denatured FXIIIa (dFXIIIa, 10 U mL^{-1}), FXIII or FXIIIa (both 10 U mL^{-1}), recombinant FXIII (rFXIII) or activated recombinant FXIII (rFXIIIa) (both 200 μg mL^{-1}) or fibrinogen (200 μg mL^{-1}) for 12 h at 4 °C. Unbound protein was removed by washes in phosphate-buffered saline (PBS), and uncoated surfaces were blocked by overlay with heat-inactivated human serum albumin (HSA; 5% in PBS) for 30 min at

20 °C [14]. Washed platelets (5×10^7 platelets mL^{-1}) were allowed to adhere for 1 h at 37 °C unless stated otherwise. In some experiments, platelets were preincubated with U73122 (5 μM) to inhibit PLC, BAPTA-AM (20 μM) to chelate intracellular Ca^{2+}, apyrase (1 U mL^{-1}) to scavenge ADP, indomethacin (10 μM) to block thromboxane A_2 synthesis, PP1 (20 μM) to inhibit Src-family kinases, GSNO (10 μM) to inhibit platelet activation, RGDS (500 μM) to broadly inhibit platelet integrins, tirofiban (2 μM) to specifically inhibit $\alpha_{IIb}\beta_3$, or LM609 (20 μg mL^{-1}) to specifically inhibit $\alpha_v\beta_3$. Incubations were performed for 20 min at 37 °C prior to platelet adhesion. In other experiments, coated wells were overlaid with polyclonal anti-fibrinogen or anti-FXIII-A blocking antibodies or control IgG (all 30 μg mL^{-1} in 10% HSA) for 30 min. Wells were washed, platelets were allowed to adhere, and adherent platelets were fluorescently stained for 1 h with tetramethyl rhodamine isothiocyanate–phalloidin. The slides were observed with an IX71 fluorescence microscope equipped with an XM10 CCD camera (Olympus, Tokyo, Japan). Images were captured under × 60 magnification and analyzed with ImageJ software (NIH, Bethesda, MD, USA).

Measurement of platelet adhesion under flow

Platelets (2×10^8 platelets mL^{-1}) were stained with $DIOC_6$ (1 μM) at 37 °C for 10 min reconstituted with autologous washed RBCs (50% v/v). Flow studies were performed with glass microslide capillary tubes (Camlab, Cambridge, UK), coated with FXIIIa (1–10 U mL^{-1}) for 12 h in the presence or absence of VWF (20 μg mL^{-1}) and blocked with bovine serum albumin (BSA) (10 mg mL^{-1}) for 1 h. To investigate how FXIIIa bound to collagen and influenced its ability to support platelet arrest, collagen (100 μg mL^{-1}) was initially incubated with FXIIIa (10 U mL^{-1}) for 15 min at 37 °C prior to immobilization. Platelets were perfused through coated tubes at the desired shear rate for 4 min. Images of stably adhered platelets and thrombi were captured by fluorescence microscopy and analyzed with ImageJ software. Data for adhesion under flow are presented as surface area coverage (%), as the software could not fully discriminate between single platelets and platelet aggregates. Measurement of thrombus volume was performed as previously described [15]. Briefly, images of platelet thrombi were captured by fluorescence and stored in a 24-bit black and white format. Thrombus surface area values were then multiplied by their corresponding mean gray values, which coincided with fluorescence intensity. The product was considered as thrombus volume and measured in arbitrary units [15–17].

Immunoprecipitation and immunoblotting

Six-well culture plates were coated overnight with dFXIIIa (10 U mL^{-1}) or FXIIIa (10 U mL^{-1}). Uncoated surfaces were blocked by overlay with HSA (5%) [14] for 30 min at 20 °C. Washed platelets (5×10^8 platelets mL^{-1}) were allowed to adhere to dFXIIIa-coated or FXIIIa-coated wells for 45 min at 37 °C in the presence or absence of receptor antagonists. Non-adherent platelets were removed, and adherent platelets were lysed with Laemmli buffer to produce whole cell lysates, or lysis buffer for immunoprecipitation studies [13]. Syk, LAT, PLCγ2 and SLP-76 were then immunoprecipitated as previously described [13]. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, immunoblotted with the required antibodies (all 1 : 1000), and then visualized by enhanced chemiluminescence.

Statistical analysis

Results are expressed as means ± standard deviations, and were analyzed with Student's *t*-test or ANOVA for unpaired data as appropriate. $P < 0.05$ and $P < 0.01$ were considered to indicate statistical significance.

Results

FXIII stimulates spreading of adherent platelets

We first characterized the ability of immobilized FXIII to support platelet adhesion and spreading under static conditions. Plasma FXIII, in both native and activated states, supported equal numbers of adherent platelets (782 ± 108 and 712 ± 82 platelets/0.1 mm^2 for FXIII and FXIIIa, respectively, $P > 0.05$) (Fig. 1Aii). At identical concentrations of the A-subunit, adhesion levels were equally reproducible with recombinant zymogen and activated enzyme forms (548 ± 92 vs. 608 ± 155 platelets/0.1 mm^2 for rFXIII and rFXIIIa, respectively, $P > 0.05$) (Fig. 1Aii). Platelets did not bind denatured FXIIIa, suggesting a dependence on its correct conformation. Moreover, consistent with previous reports, platelets did not bind the HSA used to effectively block uncoated glass surfaces [14]. Platelet adhesion to both FXIII and FXIIIa was concentration-dependent, and increased as a function of time, with similar kinetics for zymogen and activated enzyme forms (not shown).

We observed that adherent platelets on activated and zymogen FXIII had a fully spread morphology (Fig. 1A), exhibiting both filopodia and lamellipodia formation. At 60 min, the majority of platelets were fully spread, with average platelet surface areas of 24 ± 5 and 28 ± 3 μm^2 ($P > 0.05$) for FXIII and FXIIIa, respectively (Fig. 1Aiii). Similar activation-triggered shape changes were observed in platelets bound to rFXIII and rFXIIIa (Fig. 1Ai). Thus, under static conditions, immobilized FXIII and FXIIIa supported equal levels of platelet adhesion, but also similarly triggered activation and spreading in adherent platelets.

As FXIII can associate with fibrinogen in plasma [18], we examined our source of plasma FXIII for contaminating fibrinogen. Immunoblotting of fibrogammin P with a polyclonal anti-fibrinogen antibody failed to reveal the presence of fibrinogen when compared to a purified fibrinogen control (Fig. S1Ai). Furthermore, we confirmed the FXIII-A content of both fibrogammin P and rFXIII-A through immunoblot-

ting with a specific antibody (Fig. S1Aii). To confirm that platelets were binding FXIIIa independently of fibrinogen, specific blocking antibodies to FXIII-A and fibrinogen were used. Platelet adhesion to FXIIIa was abolished by overlaying the immobilized protein with a polyclonal anti-FXIII-A antibody, whereas an IgG control had no effect (Fig. 1B). Importantly, overlaying FXIIIa with an anti-fibrinogen antibody failed to influence platelet adhesion to FXIIIa, but blocked adhesion to immobilized fibrinogen. Adding anti-fibrinogen antibody to platelet suspensions prior to adhesion also did not affect platelet binding to FXIIIa (not shown). These data, along with those showing equally reproducible platelet adhesion to plasma protein-free rFXIII-A (Fig. 1A), indicate that the observed adhesion and spreading events on plasma FXIII occurred independently of fibrinogen.

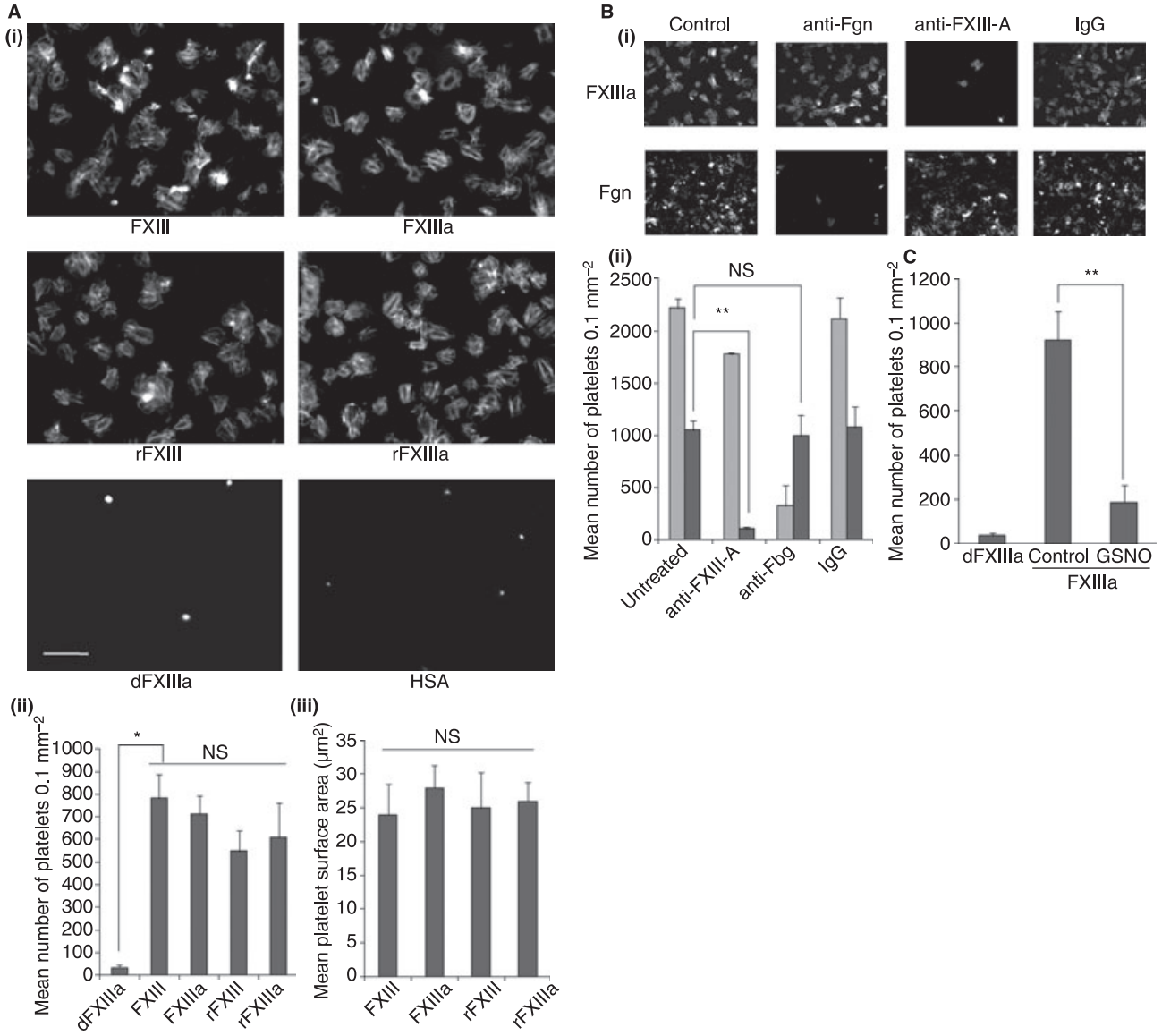

Fig. 1. Activated factor XIII (FXIIIa) stimulates spreading of adherent platelets. (A) Wells on glass slides were coated overnight at 4 °C with FXIII or FXIIIa (10 U mL^{-1}), recombinant FXIII (rFXIII) or recombinant FXIIIa (rFXIIIa) (200 μg mL^{-1}), or heat-denatured FXIIIa (dFXIIIa) (10 U mL^{-1}). Uncoated surfaces were blocked by overlay with heat-inactivated human serum albumin (HSA) (5%) for 30 min at 20 °C. Washed platelets (5×10^7 platelets mL^{-1}) were allowed to adhere for 1 h, stained with tetramethyl rhodamine isothiocyanate–phalloidin, and viewed under × 60 magnification. (i) Representative images are shown. Bar: 20 μm. (ii) Platelets were counted and expressed as number of adherent platelets per 0.1 mm^2. (iii) Images were used to determine platelet surface area. (B) As in (A), except that some wells were separately coated with fibrinogen (200 μg mL^{-1}) (light bars) or FXIIIa (10 U mL^{-1}) (dark bars), and then overlaid with the indicated polyclonal antibody (30 μg mL^{-1}) or left untreated for 30 min at room temperature prior to platelet adhesion. (B) (i) Representative images are shown. Bar: 20 μm. (ii) Platelets were counted and expressed as number of adherent platelets per 0.1 mm^2. Results are means ± standard deviations of three separate experiments with different blood donors. (C) Platelets were pretreated with *S*-nitrosoglutathione (GSNO) (10 μmol mL^{-1}) for 20 min at 22 °C or left untreated (control), and the number of adherent platelets was determined. Results are means ± standard deviations of four separate experiments with with different blood donors. Fgn, fibrinogen; NS, not significant. $^{**}P < 0.01$, $^{*}P < 0.05$.

Our data suggested that transglutaminase activity did not play a role in FXIII-mediated platelet adhesion (Fig. 1Aii). Under conditions where the transglutaminase activity of FXIIIa was inhibited by iodoacetamide (3 mM) (Fig. S2A), platelet adhesion was unaffected (Fig. S2B). Furthermore, inhibition of a potential 'PDI-like activity' of FXIIIa [12] by incubation with bacitracin (1 mM) also failed to inhibit platelet adhesion (Fig. S2C). In contrast, adhesion was significantly diminished by inhibition of platelet activation with the nitric oxide donor GSNO (10 μmol mL^{-1}) (Fig. 1C).

FXIIIa supports platelet adhesion under flow conditions

FXIIIa predominates at sites of vascular damage and interacts with proteins of the ECM [4], thereby potentially playing a role in platelet recruitment at these sites. Therefore, for the remaining experiments, we concentrated on the activated form of the enzyme, using an in vitro flow assay [14] to assess the stringency of FXIIIa–platelet binding under physiologic levels of shear. Immobilized FXIIIa, but not BSA, recruited flowing platelets, leading to stable aggregate formation at a venous shear rate of 300 s^{-1} (Fig. 2). This was maintained at arterial shear (800 s^{-1}), albeit to a significantly lower extent than at 300 s^{-1} ($P < 0.01$) (Fig. 2). Thus, immobilized FXIIIa supports platelet adhesion under physiologic conditions of flow, in a shear-dependent manner. Under these conditions, adhesion also occurred independently of transglutaminase activity, and was reproducible with FXIII and iodoacetamide-treated FXIIIa (not shown). Importantly, platelet adhesion to FXIIIa was observed with whole blood under flow (Fig. S5 and Video S1C).

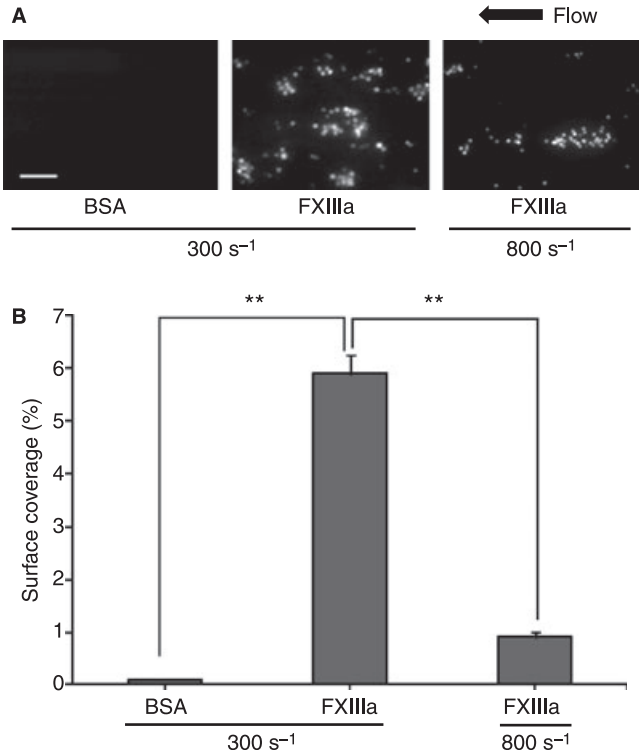

Fig. 2. Activated factor XIII (FXIIIa) supports platelet adhesion under flow. Washed platelets (2×10^8 platelets mL^{-1}) were stained with $DiOC_6$ (1 μM), reconstituted blood was perfused over immobilized FXIIIa (10 U mL^{-1}) for 4 min at the indicated shear rate, and platelet deposition was then viewed by fluorescence microscopy. Images were captured under fluorescence using CELL^P imaging software. Magnification: × 60. (A) Representative images are shown. Bar: 20 μm. (B) Data are shown as percentage area coverage and are means ± standard deviations of five separate experiments with different blood donors. BSA, bovine serum albumin. $^{**}P < 0.01$.

Roles of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ in platelet adhesion and spreading on FXIIIa

We next examined the role of integrins in platelet adhesion to FXIIIa. The specific $\alpha_{IIb}\beta_3$ antagonist tirofiban (2 μM) reduced, but did not eliminate, platelet adhesion to FXIIIa from 941 ± 42 to 566 ± 72 platelets/0.1 mm^2 ($P < 0.05$) (Fig. 3-Aii), suggesting the involvement of additional receptors. In contrast, adhesion to fibrinogen in the presence of tirofiban was almost abolished (Fig. 3C and S3). Integrin $\alpha_v\beta_3$ mediates FXIIIa binding to monocytes [19] and endothelial cells [10], and we hypothesized that it may also facilitate FXIIIa interactions with platelets. Specific inhibition of platelet $\alpha_v\beta_3$ with the blocking antibody LM609 [20] (20 μg mL^{-1}), but not IgG control, reduced platelet adhesion to FXIIIa (941 ± 42 to 480 ± 91 platelets/0.1 mm^2, $P < 0.01$). Five-fold higher concentrations of tirofiban or LM609 had no additional effect (not shown). Importantly, when used in combination, these inhibitors abolished adhesion to FXIIIa (Fig. 3Ai,ii). Inhibition of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ had divergent effects on FXIIIa-mediated platelet spreading. Whereas spreading was maintained following $\alpha_v\beta_3$ blockade (27 ± 4 μm^2 as compared with 31 ± 1 μm^2 for the presence and absence of LM609 [$P > 0.05$]), it was abolished following inhibition of $\alpha_{IIb}\beta_3$ (8 ± 2 μm^2 as compared with 31 ± 1 μm^2 for the presence and absence of tirofiban [$P < 0.05$]) (Fig. 3Aiii). Consistent with the experiments performed under static conditions, under flow, FXIIIa-mediated adhesion was reduced significantly by tirofiban ($P < 0.01$) or LM609 ($P < 0.01$), and abolished when they were used in combination (Fig. 3B). As with the IgG control, treating platelets with a blocking antibody against glycoprotein (GP)1b, Mab6D1 (20 μg mL^{-1}), had no effect on adhesion to FXIIIa under either static or flow conditions (not shown). These findings suggest that platelet adhesion to FXIIIa is dually dependent on $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$, whereas platelet spreading and potentially stable adhesion are driven by $\alpha_{IIb}\beta_3$. In contrast to the results obtained with FXIIIa, inhibition of $\alpha_v\beta_3$ had no effect on adhesion to fibrinogen (Fig. 3C). As the two proteins can associate in plasma, we investigated whether their differential integrin binding influenced platelet accrual. Fibrinogen-bound FXIII dose-dependently increased platelet recruitment by immobilized fibrinogen under physiologic flow (Fig. 3D). Thus, FXIII can enhance fibrinogen-mediated platelet accrual.

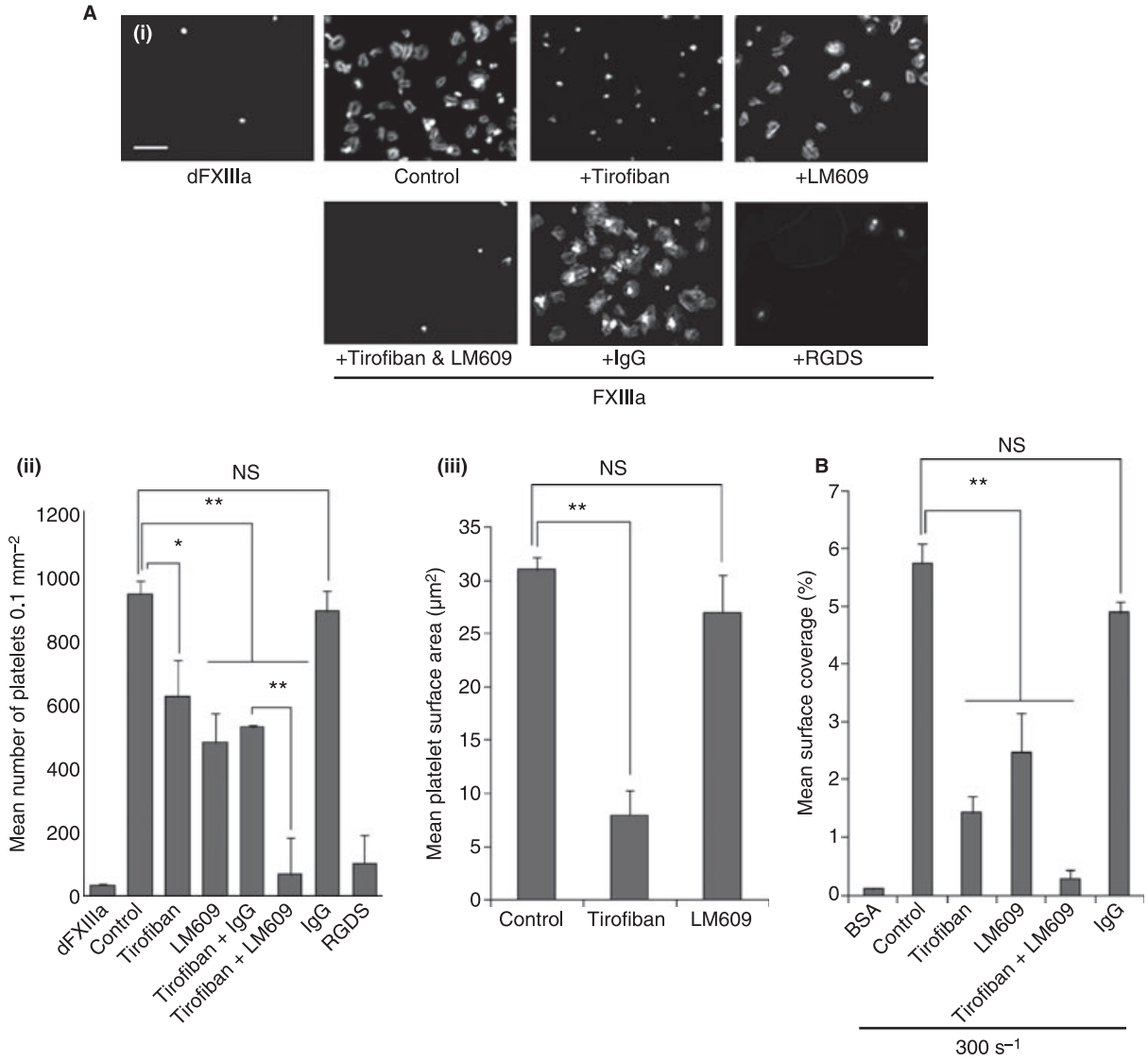

Fig. 3. Platelet adhesion to activated factor XIII (FXIIIa) is dually dependent on $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$. (A) Washed platelets (5×10^7 platelets mL^{-1}) were treated with tirofiban (2 μM), LM609 (20 μg mL^{-1}), IgG control antibody (20 μg mL^{-1}), or H-Arg-Gly-Asp-Ser-OH (RGDS) (500 μM), or left untreated (control), for 20 min, allowed to adhere to immobilized FXIIIa or heat-denatured FXIIIa (dFXIIIa) (both 10 U mL^{-1}) for 1 h at 37 °C, stained with tetramethyl rhodamine isothiocyanate–phalloidin, and viewed by fluorescence microscopy. (i) Representative images taken under × 60 magnification. Bar: 20 μm. (ii) Platelets were counted and expressed as number of adherent platelets per 0.1 mm^2. (iii) Images were used to determine platelet surface area. The results are from three separate experiments with different blood donors. (B) Washed platelets (2×10^8 platelets mL^{-1}) were treated with the appropriate receptor antagonists and stained with $DiOC_6$ (1 μM), and reconstituted blood was perfused over immobilized FXIIIa for 4 min at a shear rate of 300 s^{-1}. Images were used to calculate platelet surface area coverage. (C) As in (A), except that platelets were allowed to adhere to immobilized fibrinogen (200 μg mL^{-1}). The results are from three separate experiments with different blood donors. (D) As in (B), except that FXIII (5 and 10 U mL^{-1}) or heat-denatured FXIII (dFXIII) (10 U mL^{-1}) were allowed to bind to fibrinogen (1 mg mL^{-1}) through a 15-min preincubation at 37 °C prior to coating of tubes. Uncoated surfaces were blocked by overlay with bovine serum albumin (BSA) (10 mg mL^{-1}) for 30 min at 20 °C prior to blood perfusion at 800 s^{-1} for 4 min. The results are from three separate experiments with different blood donors. Fbg, fibrinogen; HSA, human serum albumin; NS, not significant. $^{**}P < 0.01$, $^{*}P < 0.05$.

FXIIIa supports adhesion and spreading through a Src-kinase-dependent pathway

Stable platelet adhesion and spreading via $\alpha_{IIb}\beta_3$ requires tyrosine kinase-dependent signals involving Src and Syk [21]. We examined the role of the signaling proteins required for outside-in signaling. Consistent with a tyrosine kinase-dependent mechanism, the Src-family kinase inhibitor PP1 (20 μM), but not its inactive analog (not shown), reduced platelet adhesion to FXIIIa from 762 ± 40 to 143 ± 77 platelets/0.1 mm^2 ($P < 0.01$) and abolished spreading (27 ± 1 to 11 ± 3 μm^2; $P < 0.01$) (Fig. 4A). Furthermore, adhesion to FXIIIa increased tyrosine phosphorylation of a broad range of proteins in whole cell lysates from FXIIIa-adherent platelets, with the most prominent bands being observed at 27, 36, 40, 48, 55, 72 and 130 kDa (Fig. 4B, arrows). These phosphorylation events were abolished by RGDS, consistent with integrin-mediated signaling. Using immunoprecipitation, we found that

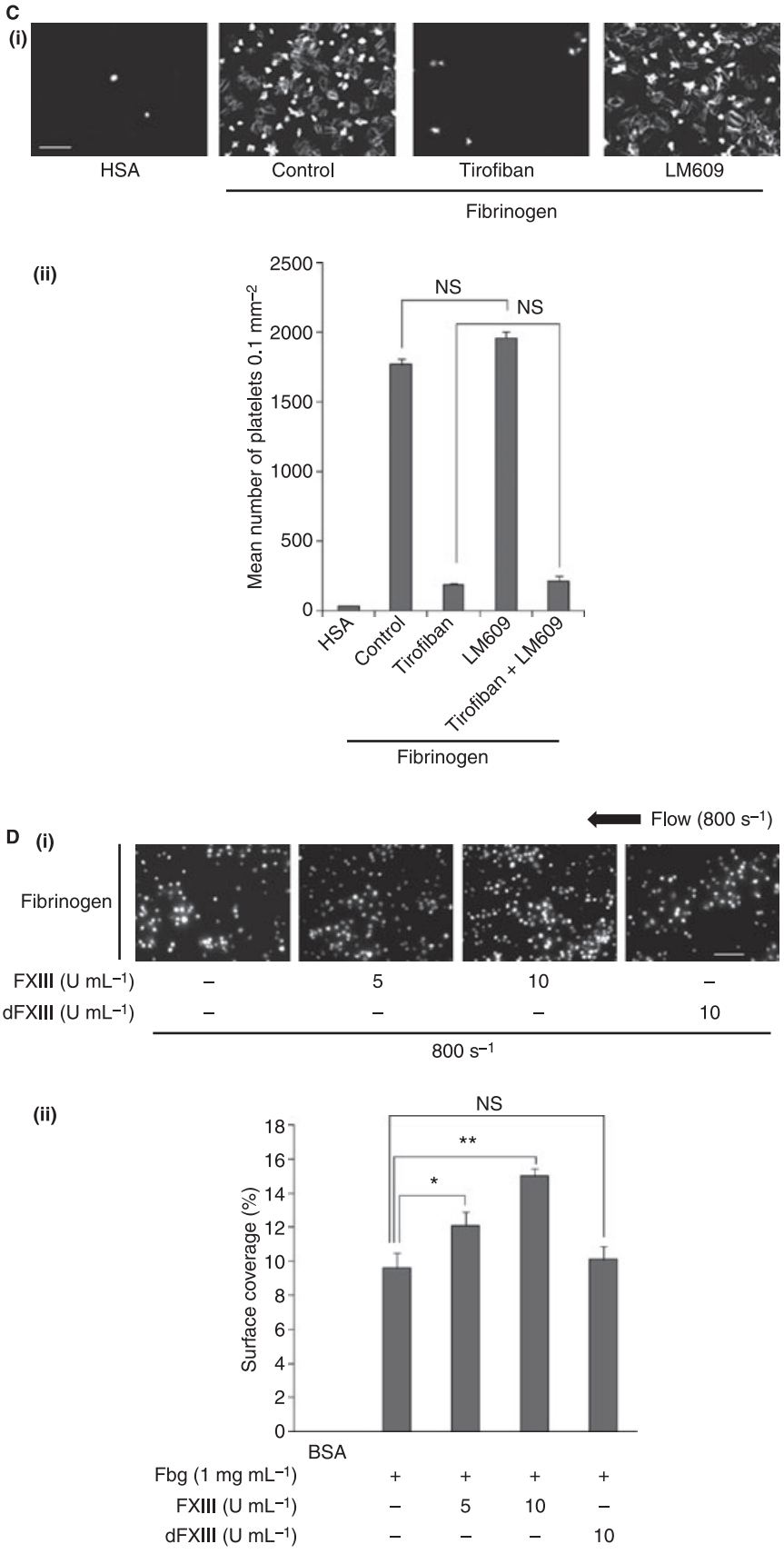

Fig. 3. Continued

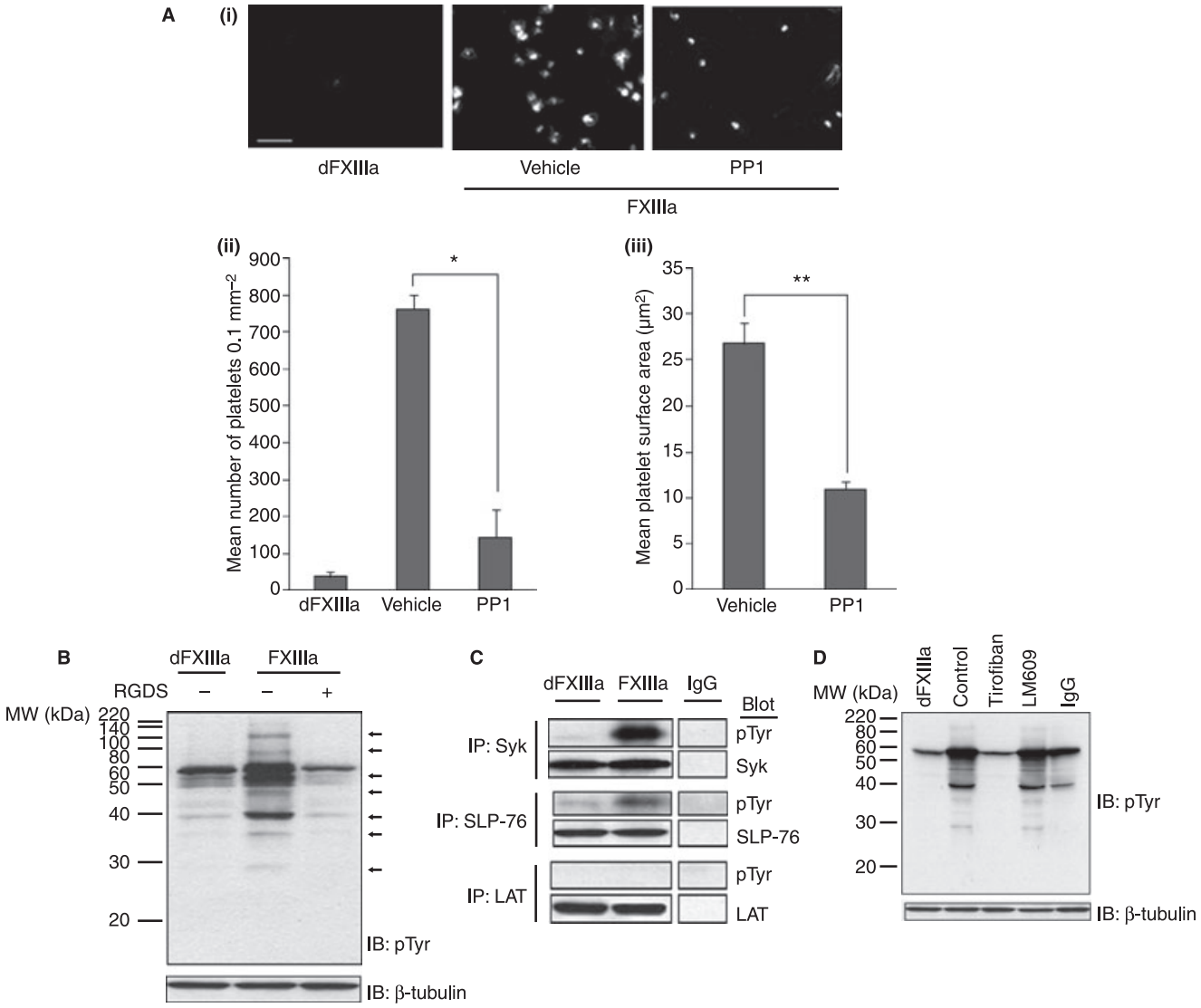

Fig. 4. Platelet adhesion and spreading on activated factor XIII (FXIIIa) is tyrosine kinase-dependent. (A) Washed platelets (5×10^7 platelets mL^{-1}) were treated with 4-amino-5-(4-methylphenyl-7-(t-butyl)pyrazolo(3,4-d)pyrimidine (PP1) (20 μM) or 0.01% dimethylsulfoxide (vehicle control) for 20 min, allowed to adherefor 1 h to immobilized FXIIIa (10 U mL^{-1}), and viewed by fluorescence microscopy. (i) Representative images taken under × 60 magnification. Bar: 20 μm. (ii) Adherent platelets were counted and expressed as number of adherent platelets per 0.1 mm^2. (iii) Images were used to determine platelet surface area. The results are from four separate experiments with different blood donors. $^*P < 0.05$, $^{**}P < 0.01$. (B) Washed platelets (5×10^8 platelets mL^{-1}) were allowed to adhere to immobilized FXIIIa (10 U mL^{-1}) or heat-denatured FXIIIa (dFXIIIa) (10 U mL^{-1}) in the presence of apyrase (1 U mL^{-1}) and indomethacin (10 μM) for 45 min at 37 °C. In some cases, platelets were treated with H-Arg-Gly-Asp-Ser-OH (RGDS) (500 μM) before adhesion. Adherent-platelet lysates were immunoblotted for phosphotyrosine (pTyr). The results are from four separate experiments with different blood donors. (C) As in (B), except that Syk, SLP-76 and LAT were immunoprecipitated from lysates, separated by SDS-PAGE, and immunoblotted against each respective protein and phosphotyrosine. The results are from three separate experiments with different blood donors. (D) As in (B), except that platelets were treated with tirofiban (2 μM), LM609 (20 μg mL^{-1}), or IgG control antibody (20 μg mL^{-1}), or left untreated (control), for 20 min at 37 °C prior to adhesion to FXIIIa (10 U mL^{-1}). The blot is representative of three separate experiments with different blood donors. IB, immunoblotting; IP, immunoprecipitation.

adhesion to FXIIIa stimulated tyrosine phosphorylation of Syk and its downstream target SLP-76 (Fig. 4C), two proteins that play critical roles in signaling by $\alpha_{IIb}\beta_3$ [21]. In contrast, LAT remained in its unphosphorylated state, distinguishing the signaling cascade from that downstream of GPVI [22] (not shown). We could not detect phosphorylation of tyrosine residues when platelets were stirred with FXIIIa in suspension (not shown). To dissect the roles of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ in signaling events induced by immobilized FXIIIa, we compared the pattern of tyrosine phosphorylation of whole cell lysates from adherent platelets treated with tirofiban and LM609. Tyrosine phosphorylation of platelet proteins was unaffected by inhibition of $\alpha_v\beta_3$, whereas blockade of $\alpha_{IIb}\beta_3$ restricted it to basal levels (Fig. 4D).

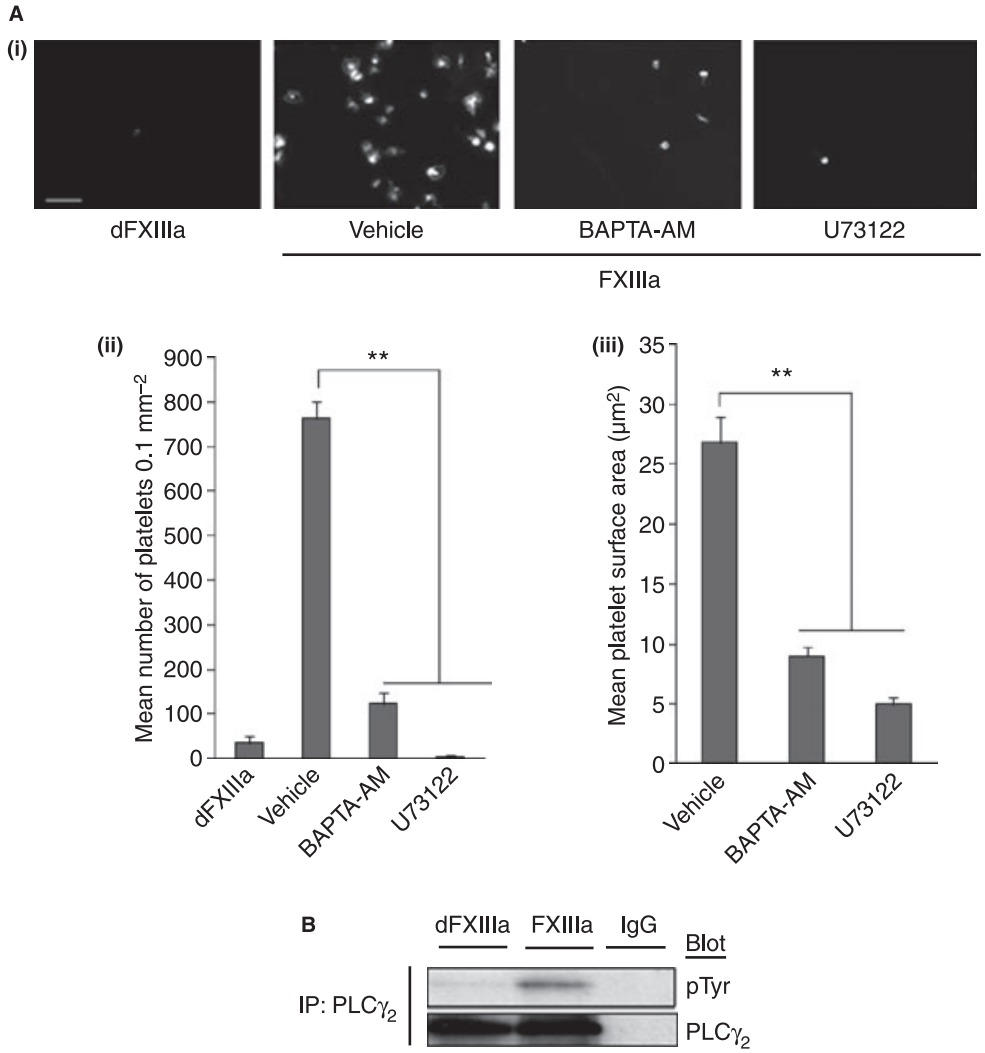

Fig. 5. Phospholipase C (PLC)γ2-mediated intracellular Ca^{2+} mobilization is required for stable platelet adhesion and spreading on activated factor XIII (FXIIIa). (A) (i) Washed platelets (5×10^7 platelets mL^{-1}) were treated with 1,2-bis-(*o*-aminophenoxy)ethane-tetra-acetic acid tetra-(acetoxymethyl) ester (BAPTA-AM) (20 μM) or U73122 (5 μM) for 20 min, allowed to adhere to immobilized FXIIIa (10 U mL^{-1}) for 1 h, stained with tetramethyl rhodamine isothiocyanate–phalloidin, and viewed by fluorescence microscopy. Bar: 20 μm. (ii) Platelets were counted and expressed as number of adherent platelets per 0.1 mm^2. (iii) Surface areas of adherent platelets were measured with ImageJ software. The results are from four separate experiments with different blood donors. $^{**}P < 0.01$. (B) Washed platelets (5×10^8 platelets mL^{-1}) were allowed to adhere to the wells of a six-well plate coated with heat-denatured FXIIIa (dFXIIIa) (10 U mL^{-1}) or FXIIIa (10 U mL^{-1}) in the presence of apyrase (1 U mL^{-1}) and indomethacin (10 μM) for 45 min. PLCγ2 was immunoprecipitated from adherent-platelet lysates, and immunoblotted for PLCγ2 and phosphotyrosine (pTyr). Blots are representative of three separate experiments with different blood donors. IP, immunoprecipitation.

FXIIIa-mediated platelet adhesion and spreading requires the mobilization of intracellular Ca^{2+}

As the mobilization of intracellular Ca^{2+} through PLCγ2 is vital for integrin-mediated adhesion and spreading [23], we examined its role in FXIIIa-stimulated platelet adhesion. The intracellular Ca^{2+} chelator BAPTA-AM (20 μM) significantly reduced platelet adhesion to FXIIIa from 762 ± 40 to 123 ± 26 platelets/0.1 mm^2 ($P < 0.01$) (Fig. 5Aii) and prevented spreading. We next used the PLC inhibitor U73122 (5 μM), which completely abolished stable platelet adhesion (Fig. 5Aii). Consistent with these data, PLCγ2 was tyrosine-phosphorylated, suggesting its activation [24], when platelets adhered to FXIIIa (Fig. 5B). These data suggest that adhesion to FXIIIa leads to activation of PLCγ2, which mobilizes the intracellular Ca^{2+} that is required for spreading.

FXIIIa enhances platelet recruitment by the ECM under arterial flow conditions

The exposure of ECM proteins such as collagen and collagen-bound VWF at sites of vascular injury leads to rapid activation

and retention of platelets [25]. We hypothesized that, as FXIIIa could support adhesion alone, it may supplement platelet recruitment by other matrix proteins. We co-immobilized a fixed concentration of VWF (20 μg mL^{-1}) with a range of FXIIIa concentrations (1–10 U mL^{-1}). At a shear rate of 800 s^{-1}, immobilized VWF (20 μg mL^{-1}) supported adhesion of single platelets that covered 8.9% ± 0.1% of the VWF-coated surface. However, platelet surface coverage was increased significantly when VWF was immobilized together with FXIIIa (Fig. 6). Maximal effects were observed with FXIIIa (10 U mL^{-1}), where surface area coverage was increased from 8.9% ± 0.1% (VWF alone) to 15.0% ± 1.6% ($P < 0.05$). Importantly, there was a clear contrast between single platelets bound on a VWF surface and platelet aggregates observed on a VWF/FXIIIa surface (Fig. 6A and Video S1A).

Factor XIII binds to collagen [26], which may allow FXIIIa to be immobilized at sites of vascular damage, thereby promoting platelet adhesion. Using methodology provided in the data supplement (Data S1), we observed that FXIIIa, but not zymogen FXIII, bound to collagen (Fig. S4), and then examined how this influenced platelet adhesion to collagen. Under our experimental conditions, collagen (100 μg mL^{-1}) alone supported the deposition of robust platelet-rich thrombi, resulting in a mean thrombus volume of (3.7 ± 0.1) × 10^5 arbitrary units. Importantly, collagen-bound FXIIIa facilitated a significant increase in the volumes of stable thrombi to (6.7 ± 0.5) × 10^5 arbitrary units ($P < 0.05$) (Fig. 7C), without influencing surface coverage (Fig. 7B). Crucially, this required the correct protein folding of FXIIIa, as heat-denatured FXIIIa could not enhance the effects of collagen ($P > 0.05$).

Discussion

Although associations between platelets and FXIIIa are recognized, the physiologic consequences of these interactions are poorly defined. The aims of our study were three-fold: first, to investigate the ability of immobilized FXIIIa to support adhesion and spreading under static and flow conditions; second, to determine the molecular mechanisms underpinning platelet adhesion; and third, to examine whether FXIIIa could act cooperatively with other thrombogenic proteins to enhance platelet accrual under flow. Our results confirm those of Dardik *et al*. [10], who showed that platelets could bind both FXIII and FXIIIa. However, the

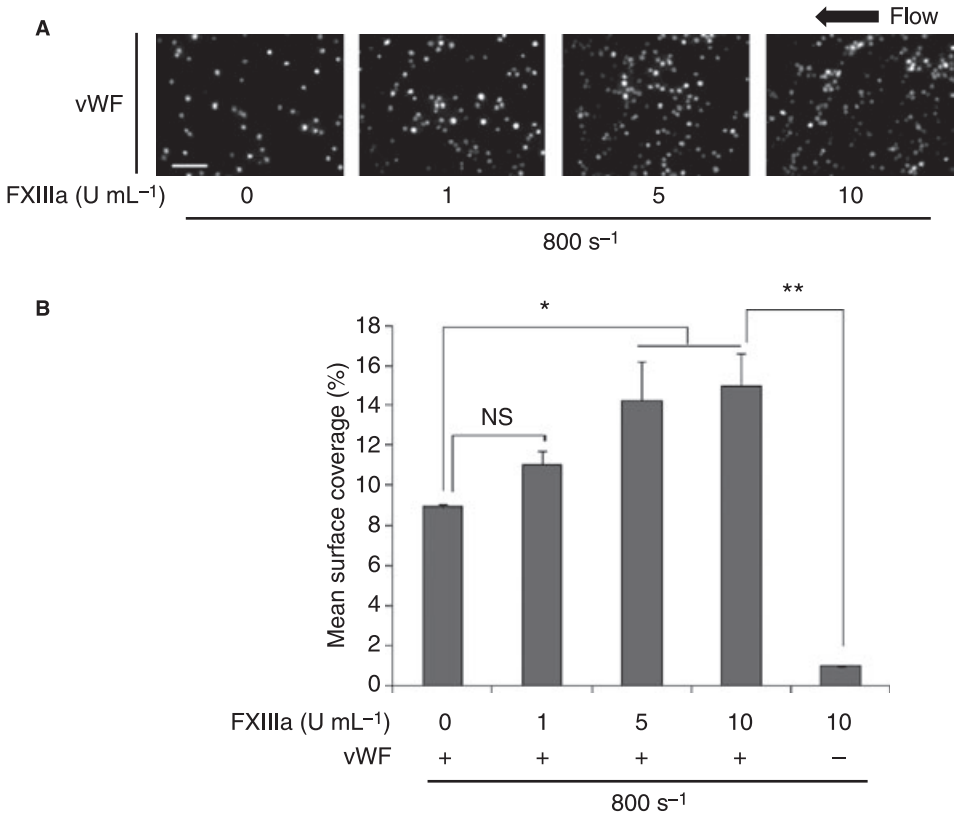

Fig. 6. Activated factor XIII (FXIIIa) enhances platelet thrombus formation by von Willebrand factor (VWF). Reconstituted blood was perfused over immobilized FXIIIa (10 U mL^{-1}) or VWF (20 μg mL^{-1}) either alone or co-immobilized with increasing concentrations of FXIIIa (1–10 U mL^{-1}) for 4 min at 800 s^{-1}, and then viewed by fluorescence microscopy. Images from eight random fields of view were captured under fluorescence, with CELL·P imaging software (magnification: × 60). (A) Representative images are shown. Scale bar: 20 μm. (B) Data shown are means ± standard deviations of three separate experiments with different blood donors. NS, not significant. $^{**}P < 0.01$, $^{*}P < 0.05$.

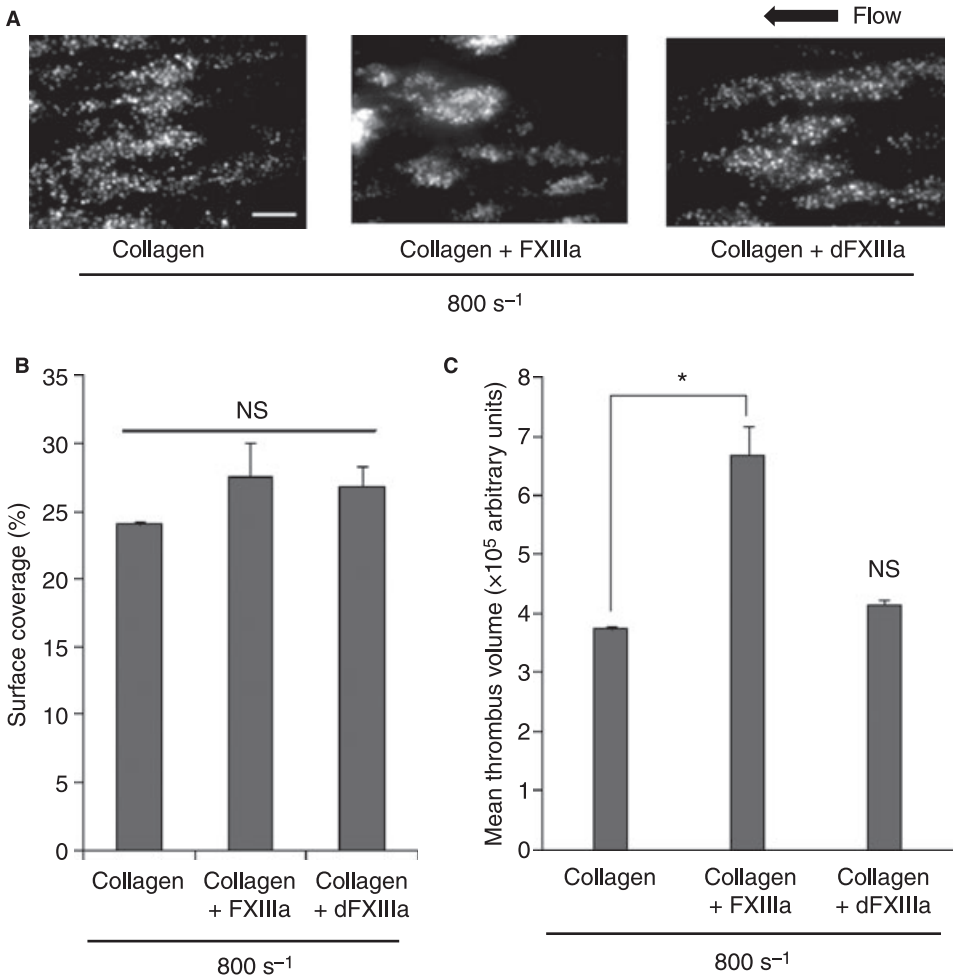

Fig. 7. Activated factor XIII (FXIIIa) is immobilized on collagen and enhances its thrombogenic capacity. FXIIIa (10 U mL^{-1}) or heat-denatured FXIIIa (dFXIIIa) (10 U mL^{-1}) were bound to collagen (100 μg mL^{-1}) through a 15-min preincubation at 37 °C prior to coating of tubes. Reconstituted blood was perfused through the coated tubes for 4 min at 800 s^{-1}, and thrombus formation was viewed by fluorescence microscopy. (A) Representative images of stable thrombi formed on each surface are shown. Bar: 20 μm. (B) ImageJ software was used to measure percentage surface area coverage. (C) Volumes of thrombi formed on each surface were measured as previously described [15]. The results are from four separate experiments with different blood donors. NS, not significant. *$P < 0.05$.

data presented here also greatly extend that work by demonstrating several key factors, including the fact that FXIIIa supports adhesion independently of transglutaminase and PDI-like activity, induces a tyrosine kinase signaling pathway downstream of $\alpha_{IIb}\beta_3$, and supports platelet accrual under physiologic conditions of flow.

Platelet adhesion and spreading on FXIIIa occur through collaborative binding by $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$. Previous studies used the antibody 7E3 [7], or its humanized derivative abciximab [10], and eptifibatide [27] in attempt to specifically demonstrate the critical role of $\alpha_{IIb}\beta_3$ in binding FXIIIa. However, unlike tirofiban, all three inhibitors also block $\alpha_v\beta_3$ [28]. Thus, conclusions from these studies overlooked the potential contribution of $\alpha_v\beta_3$. Using tirofiban for its strict specificity for $\alpha_{IIb}\beta_3$, we found that this integrin was only partially responsible for platelet binding to FXIIIa, with LM609 revealing that $\alpha_v\beta_3$ was needed for maximal adhesion. Platelet adhesion to FXIIIa was only effectively abolished when both integrins were blocked. It is noteworthy that platelet $\alpha_v\beta_3$ has been shown to possess an affinity 150-fold greater than $\alpha_{IIb}\beta_3$ for at least one common ligand [29]. Thus, despite its lower copy number, it is an important mediator of platelet adhesive events. Although FXIIIa-mediated adhesion depends on both receptors, platelet spreading, required for stable adhesion under flow, is mediated solely by $\alpha_{IIb}\beta_3$. Together, these data suggest a model whereby $\alpha_{IIb}\beta_3$ reinforces $\alpha_v\beta_3$-mediated platelet adhesion to FXIIIa through signaling events that lead to spreading. FXIIIa stimulates platelet spreading through a tyrosine kinase-dependent mechanism downstream of $\alpha_{IIb}\beta_3$, resulting in the tyrosine phosphorylation and activation of a number of proteins, including Syk, SLP-76, and PLCγ2, leading to the Ca^{2+} mobilization required for platelet activa-

tion. Consistent with this, platelet inhibition by GSNO markedly reduced stable adhesive contacts with FXIIIa. In contrast, $\alpha_v\beta_3$ did not participate in the signaling events, in line with its redundancy in the spreading process. Despite the ability of FXIIIa to stimulate spreading, it is notable that it did not induce aggregation or tyrosine phosphorylation in platelet suspensions (not shown). This explains, in part, why previous studies have not reported signaling events induced by this enzyme. Furthermore, signal transduction induced solely by immobilized FXIIIa may suggest a unique mechanism that allows it to promote platelet activation only when it is localized at sites of vascular damage. Thus, in addition to identifying a potentially new platelet receptor for FXIIIa, we provide the first evidence that FXIIIa activates platelets through tyrosine kinase-dependent signaling.

It is possible that FXIIIa, through mediating both platelet adhesion and activation, could contribute to platelet recruitment at sites of vascular injury. In plasma, FXIII can associate with fibrinogen, an important ligand for $\alpha_{IIb}\beta_3$. We demonstrate that platelets can bind FXIII independently of fibrinogen, as we excluded its presence from our plasma-purified FXIII preparation, and platelets adhered to plasma protein-free recombinant FXIII-A. Binding to FXIII, but not to fibrinogen, was reduced by inhibition of $\alpha_v\beta_3$. This is consistent with descriptions of a minor platelet role for $\alpha_v\beta_3$ in binding fibrinogen [29]. Importantly, it suggests that different, non-redundant mechanisms control platelet binding to the two proteins. In fact, we observed that fibrinogen-bound FXIII increased platelet binding to immobilized fibrinogen under physiologic flow, suggesting that FXIII can collaborate with fibrinogen in mediating platelet accrual. Although these data appear to contradict those of Nagy *et al.* [27], who reported that platelet–FXIII interactions were wholly dependent on fibrinogen, they are supported by a report from Cox and Devine [7], who also demonstrated that washed platelets and, indeed, purified $\alpha_{IIb}\beta_3$ could bind FXIIIa in the absence of fibrinogen, with this being enhanced by its presence. Nevertheless, both studies examined interactions with platelets in suspension using flow cytomery, whereas immobilized FXIIIa was used in the present study, which limits comparison of results.

Under high shear, VWF-mediated platelet tethering retards flowing platelets, allowing their activation in response to exposed collagen [25]. Platelet adhesion is then strengthened by integrin-mediated spreading, which is facilitated by various plasma proteins. We observed that, at high shear, the presence of surface-immobilized FXIIIa led to platelet aggregate formation, which was unobservable on VWF alone, indicating that FXIIIa can act in concert with VWF to arrest flowing platelets. Perhaps more importantly, FXIIIa bound to collagen enhanced the ability of this key matrix protein to form platelet-rich thrombi. By binding collagen exposed at sites of vascular injury, FXIIIa, like VWF, may increase platelet recruitment by supplementing the number of platelet-binding sites available per collagen fiber. This enhances the thrombotic potential of collagen by supplementing activatory signal transduction through the long-lived, integrin-mediated 'outside-in' signaling that is essential for maintaining interplatelet contacts within platelet aggregates and initiating clot retraction [25]. Thus, FXIIIa promotes the thrombogenic effects of key ECM proteins, thereby illustrating its potential role alongside other plasma and matrix proteins in propagating platelet activation at sites of vascular damage.

The present study raises the possibility of a physiologic role for FXIIIa in supporting platelet accrual and spreading through multiple integrins. Although FXIIIa does not induce platelet aggregation, FXIII-A-deficient mice exhibit elongated tail bleeding times [30] and markedly diminished clot retraction [31], suggesting a potential role for FXIII in platelet-dependent thrombosis. In humans, FXIII(a) involvement in platelet aggregation is less clear. Whereas investigators in an early study found that platelets aggregated normally in response to ADP, ristocetin, and collagen [32], a more recent study revealed diminished responses to ADP and epinephrine by platelets from three FXIII-A-deficient patients [33]. We speculate that the interaction of platelets with FXIIIa may have particular relevance at vascular injury sites, where it could potentiate the thrombogenic effects of exposed collagen and VWF and immobilized fibrinogen. Recent evidence from large clinical studies suggests that elevated plasma FXIII antigen and activity levels may be risk factors for myocardial infarction [34] and peripheral vascular disease (PVD) [35]. Taken together with the increasingly established involvement of platelets in the pathogenesis of cardiovascular disease [25], observations that patients with PVD have circulating platelets bound to more FXIII than healthy controls [36] suggest that platelet–FXIII(a) interactions may have pathologic consequences in humans. Thus, we identify FXIII/FXIIIa as a novel component of the growing numbers of proteins, including CD40, thrombospondin, FXI and protein C, localized to areas of vascular damage, that activate blood platelets through multiple integrated signaling pathways. Like FXIIIa, such proteins may play modest roles in platelet-mediated primary hemostasis, but they may contribute significantly to vascular occlusive pathologies. The involvement of platelet–FXIIIa interactions in pathologic thrombosis thus requires further investigation.

Acknowledgements

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Disclosure of Conflict of Interest

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Purity and FXIII-A content of fibrogammin P and rFXIII-A.
Figure S2. Platelet adhesion to FXIIIa is independent of transglutaminase and PDI activity.
Figure S3. Differential effects of tirofiban on platelet adhesion to fibrinogen and FXIIIa.
Figure S4. FXIIIa but not FXIII immobilizes rapidly on collagen.
Figure S5. Platelets in whole blood adhere to FXIII under flow.
Video S1. FXIIIa enhances VWF-mediated and collagen-mediated platelet-thrombus formation under flow.
Data S1. Methods.

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Accompanying Videos: Factor XIII supports platelet thrombus formation under flow in an integrin-dependent manner.

Video 1: Glass microslide capillary tubes were coated with activated Factor XIII (FXIIIa, 200µg/mL) for 12h at 4°C. Uncoated surfaces were blocked with bovine serum albumin (10mg/mL). Washed platelets were reconstituted with autologous red blood cells (RBC) to final concentrations of 2×10^8 platelets/mL and 50% (v/v) respectively, stained with $DiOC_6$ (1µmol/L) and perfused through the tubes for 4min at a shear rate of $300s^{-1}$ to simulate venous blood flow. Videos of adherent platelets were captured using the ×60 objective lens of an IX71 fluorescence microscope coupled with an XM10 CCD digital camera (Olympus, Japan). **Video 2:** As in video 1, except platelet/RBC suspension was perfused at $800s^{-1}$ to simulate arterial flow. **Video 3:** Capillary tubes were coated with recombinant Factor XIII A-subunit (rFXIII-A, 200µg/mL) for 12 hours at 4°C. Whole blood was treated with Phe-Pro-Arg-Chloromethylketone (40µmol/L) and incubated with $DiOC_6$ (1µmol/L) prior to perfusion through the tubes for 4min at a shear rate of $300s^{-1}$. **Video 4:** As in video 1, except platelets were preincubated with the $\alpha_{IIb}\beta_3$ antagonist, tirofiban (2µM) for 20min prior to perfusion. **Video 5:** As in video 1, except platelets were preincubated with the $\alpha_v\beta_3$ antagonist LM609 (20µg/mL) for 20min prior to perfusion. All videos are representative of at least 3 experiments with separate blood donors.