BIOLOGIC PROPERTIES OF PLATELET-DERIVED MICROPARTICLES:
CHARACTERIZATION OF MECHANISMS OF GENERATION AND ADHESION

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of The Institute of Medical Science,
University of Toronto

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BIOLOGIC PROPERTIES OF PLATELET-DERIVED MICROPARTICLES: CHARACTERIZATION OF MECHANISMS OF FORMATION AND ADHESION.

Master of Science degree 2001, Nada Miscevic, Graduate Department of The Institute of Medical Science, University of Toronto.

Platelet-derived microparticles (MP) are procoagulant membrane microvesicles generated upon platelet activation and observed in thrombocytopenic and thrombotic clinical states. The mechanism of MP formation and their adhesive properties are poorly understood. We characterize the roles of fibrinogen, its binding peptides and GPIIb/IIIa receptor in thrombin induced human platelet MP formation utilizing flow cytometry. Further, we characterize and compare platelets and MP adhesion to surface-bound ligands (fibrinogen, fibrin and collagen type I) using a unique, static, microsphere based adhesion assay, flow cytometry and scanning electron microscopy. MP formation was metal ion, fibrinogen and GPIIb/IIIa dependent. Specific GPIIb/IIIa reagents (antibodies, drugs) and fibrinogen peptides (RGDS, H12) and antibodies fully abolished MP formation. MP adhesion to immobilized vascular ligands was metal-ion dependent and receptor-ligand specific. In conclusion, fibrinogen (RGDS, H12) and GPIIb/IIIa are absolutely required for thrombin induced MP generation. Further, MPs adhesion to fibrinogen, fibrin and collagen-coated surfaces is mechanistically similar to that of platelets.
Acknowledgements

This work has been supported by Fellowships from the Natural Sciences and Engineering Research Council of Canada (NSERC) and The University of Toronto, and by the University Health Network Hemostasis and Thrombosis Research Fund.

I am deeply grateful to Dr. Erik Yeo for his outstanding mentorship, guidance, scientific expertise and the given opportunity to study in the field of platelets and platelet derived microparticles. I am very grateful to Dr. Margaret Rand and Dr. Andre Schuh, members of my Programme Committee, for scientific expertise and many helpful insights and suggestions. I would also like to thank Giovanni Battista Calvieri for teaching me the art of electron microscopy.

I am thankful to my parents and my sister, who always believed in me and thought me to love the process of learning and enjoy the pathways that lead to discovery.

I would like to express deep gratitude to my husband Zoran, a science researcher at the Ontario Science Centre and to my eight years old, ever inquisitive, son Filip, for all the patience, love and encouragement. I devote this work to them.

Nada Miscevic

In Toronto, Aug 24/2001
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<tbody>
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<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ACS</td>
<td>Acute coronary syndrome</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AnV</td>
<td>Annexin V</td>
</tr>
<tr>
<td>APC</td>
<td>Activated protein C</td>
</tr>
<tr>
<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>Coll</td>
<td>Collagen</td>
</tr>
<tr>
<td>CVA</td>
<td>Cerebrovascular accidents</td>
</tr>
<tr>
<td>CM</td>
<td>Coated microspheres</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DIC</td>
<td>Disseminated intravascular coagulation</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>Fgn</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>Ftn</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Fl</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>FSC</td>
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</tr>
<tr>
<td>GMP (-140)</td>
<td>Granule-membrane protein</td>
</tr>
<tr>
<td>GP</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>G-protein</td>
<td>Guanine nucleotide-binding protein</td>
</tr>
<tr>
<td>GT</td>
<td>Glanzmann's thrombasthenia</td>
</tr>
<tr>
<td>HIT</td>
<td>Heparin induced thrombocytopenia</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HTB</td>
<td>HEPES-Tyrode's buffer</td>
</tr>
<tr>
<td>HUS</td>
<td>Hemolytic uremic syndrome</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>IC50</td>
<td>50% inhibition concentration</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>ITP</td>
<td>Idiopathic thrombocytopenic purpura</td>
</tr>
<tr>
<td>kD</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LIBS</td>
<td>Ligand induced binding site</td>
</tr>
<tr>
<td>LS</td>
<td>Light scatter</td>
</tr>
<tr>
<td>MP</td>
<td>Microparticle</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>OD50</td>
<td>50% optimum dose</td>
</tr>
<tr>
<td>OCS</td>
<td>Open canalicular system</td>
</tr>
<tr>
<td>PADGEM</td>
<td>Platelet-activation dependent granule-external membrane</td>
</tr>
<tr>
<td>PAR1</td>
<td>Protease activating receptor 1</td>
</tr>
<tr>
<td>PAR4</td>
<td>Protease activating receptor 4</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCM</td>
<td>Protein-coated microspheres</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PF3</td>
<td>Platelet factor 3</td>
</tr>
<tr>
<td>PGE₁</td>
<td>Prostaglandin E₁</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipids</td>
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<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PNH</td>
<td>Paroxysmal nocturnal hemoglobinuria</td>
</tr>
<tr>
<td>PPACK</td>
<td>D-phenylalanyl-L-propyl-arginy1 chloromethyl ketone</td>
</tr>
<tr>
<td>PPP</td>
<td>Platelet-poor plasma</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet-rich plasma</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidyl serine</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin Glycoprotein Ligand-1</td>
</tr>
<tr>
<td>PTCA</td>
<td>Percutaneous transluminal coronary angioplasty</td>
</tr>
<tr>
<td>RVVT</td>
<td>Russell Viper Venom Time</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium-dodecyl sulfate polyacrylamide-gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SFLLRN, SF</td>
<td>PAR1 thrombin receptor activating peptide</td>
</tr>
<tr>
<td>SSC</td>
<td>Side size scatter</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TIA</td>
<td>Transient ischemic attacks</td>
</tr>
<tr>
<td>TTP</td>
<td>Thrombotic thrombocytopenic purpura</td>
</tr>
<tr>
<td>TH</td>
<td>Human α-thrombin</td>
</tr>
<tr>
<td>Vtn</td>
<td>Vitronectin</td>
</tr>
<tr>
<td>vWf</td>
<td>Von Willebrand factor</td>
</tr>
<tr>
<td>UPM</td>
<td>Uncoated polystyrene (plain) microspheres</td>
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Chapter 1 Introduction

1.1 Platelets and their role in circulation

Platelets are circulating anucleated blood cells derived from the fragmentation of bone marrow megakaryocytes. Each day, 2.5x10^9 platelets are generated and circulate at a concentration of 150–350x10^9/l for 7-10 days [1]. The major role of platelets in hemostasis is to prevent blood loss by preserving vessel wall integrity [2]. This hemostatic role requires the additional contribution of the vasculature, coagulation proteins and other blood cells. The formation of a hemostatic plug is a physiologic protective mechanism, but in the setting of an altered vessel wall, as may occur in atherosclerosis, platelet thrombi may lead to thrombotic disease and precipitate death by myocardial or cerebral infarction [3].

Platelets provide a primary hemostatic plug at the sites of vascular injury by cellular adhesion, aggregation, secretion and elaboration of procoagulant surface activity and the generation of procoagulant microparticles (MPs). Each of these phases involves specific platelet proteins and biochemical pathways with transition of platelets from a quiescent circulating resting state to a hemostatically active state [2]. While we have a good understanding of platelet physiology and the role of platelets in hemostasis and thrombosis, platelet-derived microparticles remain incompletely understood. MPs are considered physiologically important as they are found in a wide range of pathophysiological states associated with platelet activation including idiopathic thrombocytopenic purpura (ITP), thrombotic thrombocytopenic purpura (TTP), myocardial infarction (MI), acute coronary syndromes (ACS), venous thrombotic event (VTE) and sepsis and have a highly
procoagulant surface [4-8]. Thus, their study is an important research area in platelet physiology, thrombosis and vascular biology.

1.2 Platelet function

1.2.1 Platelet activation and signalling

Morphologically, resting platelets are discoid shaped and 1.5-3 \( \mu \text{m} \) in diameter [9]. When they encounter: 1) damaged blood vessels with exposed subendothelial matrix (collagen, fibronectin, vitronectin, laminin); 2) immobilized fibrinogen, fibrin, von Willebrand factor (vWF), or collagen; 3) altered blood vessel wall with deposits of fibrinogen and fibrin strands; or 4) physiological agonists (thrombin, collagen, complement attack complex C5b-9, ADP, thromboxane A\(_2\) or epinephrine), they become activated [2,10-13]. Their activation is manifested by several phenomena including adhesion, aggregation, secretion, shedding of microparticles and the elaboration of surface procoagulant activity (Figure 3, pg. 20). Morphologically, they change to a spherical shape and then extend pseudopodia while attaching to adhesive surfaces, eventually spreading completely ("fried egg" shape) [9]. Biochemically, agonist stimulation, mostly through G protein-coupling (guanine nucleotide binding proteins), interposed between platelet receptors and intracellular effectors, results in membrane phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) hydrolysis, generating internal (intracellular) second messengers such as diacylglycerol (DAG) that activates protein kinase C (PKC) and inositol 1,4,5-trisphosphate (IP\(_3\)), resulting in increased cytosolic Ca\(^{2+}\) [14-16]. An increase in cytosolic Ca\(^{2+}\) promotes activation of phospholipase A\(_2\) (PLA\(_2\)), catalysing the release of arachidonic acid (AA) leading to the generation of prostaglandins (PGE\(_2\)) or thromboxanes (TxA\(_2\)) [17]. TxA\(_2\) transmigrates to the extracellular medium and acts synergistically with ADP or thrombin to stimulate other platelets, thereby amplifying the
local process of platelet activation [18]. Elevated intracellular Ca\textsuperscript{2+} released by dense tubular system promotes the phosphorylation of the myosin light chain (MLC) by the myosin light chain kinase (MLCK) activated by a Ca\textsuperscript{2+} calmodulin dependent protein kinase. These events lead to activation of the membrane glycoprotein GPI\textsubscript{b}/\textsubscript{IIa} receptor to a high affinity binding state able to bind plasma protein fibrinogen, by mechanisms that are still poorly understood, but that involves PKC [14-17]. This further leads to "outside-in" signalling, reorganisation of the platelet cytoskeleton, internal granule secretion, platelet aggregation and shedding of platelet membrane MPs [14,19,20]. Activated platelet membranes and their shed platelet MPs further provide a surface for coagulation factor assembly, contributing to coagulation and thrombin generation [15,16,21].

1.2.2 Platelet adhesion

In the circulation, the interaction of specific platelet receptors and vascular adhesive ligands involved in platelet adhesion are dictated by the conditions of blood flow [16,22]. Under conditions of low shear, such as those found in the venous circulation and in regions of stasis, platelets have the potential to bind to several surface ligands: 1) collagen, via \( \alpha_2\beta_1 \) (GPI\textsubscript{Ia}/\textsubscript{IIa}); 2) fibronectin, via \( \alpha_5\beta_1 \) (GPI\textsubscript{c}/\textsubscript{IIa}); 3) vitronectin, via \( \alpha\nu\beta_3 \) (CD51/61), 4) laminin, via \( \alpha_6\beta_1 \) (GPI\textsubscript{c}/\textsubscript{IIa}); or 5) fibrinogen, fibronectin, vitronectin through GPI\textsubscript{b}/\textsubscript{IIIa} [10,23-25]. Fibrinogen and collagen are the most adhesive vascular surface ligands under low shear forces (20-200 s\textsuperscript{-1}) [22].

In contradistinction, under conditions of high shear rates, such as those found in the arterial (300-800 s\textsuperscript{-1}), microcirculation (500-1600 s\textsuperscript{-1}) or stenotic vessels (800-10000 s\textsuperscript{-1}), platelets bind to the multimeric protein von Willebrand factor (vWF) via the GPI\textsubscript{b}/IX complex (CD42b) [18,26] or through the activated GPI\textsubscript{b}/\textsubscript{IIIa} receptor to either or both vWF
and fibrinogen) [12,27]. The interaction between vWF and the GPIb receptor is initiated by the high vascular shear forces and this complex does not participate in physiologic platelet adhesion if the shear forces in blood are low [2]. In the arterial circulation, platelets attach to a primary nidus of surface adherent platelets, in a process termed “aggregation”, forming so-called “white thrombi” [22,28]. These arterial thrombi are recognized to be more difficult to dissolve with thrombolytic therapy because of their large contingent of platelets within. As a result their generation is better controlled with agents known to affect platelet activation processes such as aspirin (that inhibits cyclooxygenase, thereby inhibits generation of thromboxanes) or novel anti-aggregating agents (ReoPro and Integrilin) that block the GPIIb/IIIa receptor [2,22].

1.2.3 Platelet aggregation

Following platelet adhesion, platelets aggregate to form a hemostatic plug or an arterial thrombus, using the layer of adherent platelets as a foundation [2,16]. Platelet aggregation requires active platelet metabolism and prior platelet stimulation by any of a number of physiological platelet agonists including thrombin, collagen, complement attack complex C5b-9, ADP, epinephrine or thromboxane A2 [13]. Activation of platelet metabolism results in the activation and a conformational change of the GPIIb/IIIa receptor that then becomes competent to bind its main binding ligand, plasma fibrinogen, resulting in “outside-in” signalling that further initiates GPIIb/IIIa receptor clustering and platelet cytoskeleton reorganisation [29,30]. Fibrinogen bound to activated GPIIb/IIIa cross-links adjacent activated platelets, resulting in platelet aggregation and platelet hemostatic plug or thrombus formation [19,31].
1.2.4 Platelet cytoskeleton

The resting platelet cytoskeleton is a network of proteins that include actin, actin-binding protein, alpha-actinin, tropomyosin, vinculin and caldesmon, and that is associated with GPIb/X glycoprotein and maintains the discoid shape of resting platelets [9]. Upon platelet activation phosphorylated myosin and talin joins the cytoskeleton. Actin monomers polymerise, forming a mass of bundled filaments connected with GPIIb/IIIa and GPIa/IIa receptors via talin [32]. During these events, platelets lose their discoid shapes, becoming more spherical and extend philopodia. Adjacent platelets aggregate via clustered GPIIb/IIIa receptors, interconnecting fibrinogen and fibrin and the cytoskeleton. This leads to a consolidated platelet plug, with clot retraction mediated by contraction of the cytoplasmomicrofilamentous actin and myosin web [9,17].

In the last steps of aggregation calpain, a Ca$^{2+}$ dependent protease, cleaves actin-binding protein and talin, once again reorganising the cytoskeleton, and contributing to microparticulization of platelet membranes and the formation of procoagulant microparticles [33,34]. The exact enzymes and substrates in the process of GPIIb/IIIa “outside-in” signalling, receptor clustering, cytoskeleton formation and disintegration, microparticle formation and clot retraction continue to be elaborated [19].

1.2.5 Platelet secretion

Upon platelet stimulation and cytoskeleton reorganisation, the contents of platelet storage granules (α-granules, dense bodies and lysosomes) move to the centre of the platelet and are released by membrane fusion with the platelet open canalicular system (OCS) and thus the external platelet membrane surface [35,36]. Alpha and dense-granules are more readily released upon platelet stimulation than are the contents of lysosomes [9,36]. The degree and
extent of secretion depends on the agonist used and its concentration. Thrombin and collagen are the strongest physiological combination that leads to the secretion of all three types of granules from platelets [16]. A similar effect is achieved in vitro with the non-physiological calcium-ionophore A23187. Contents of the α-granules are released first, followed by the dense-granules and then the lysosomes [37]. Other agonists (ADP, epinephrine, TxA₂) are considered "weaker" agonists and are able to initiate only α and dense-granules release and only partially [9,15].

Platelet α-granules contain: 1) platelet specific proteins (platelet factor 4, β-thromboglobulin family); 2) adhesive glycoproteins (fibrinogen, fibronectin, vWF, vitronectin, thrombospondin); 3) coagulation factors (factor V, protein S, factor XI); 4) mitogenic factors (platelet-derived growth factor (PDGF), transforming growth factor-β, endothelial cell growth factor, epidermal growth factor); 6) fibrinolytic inhibitors (α₂-plasmin inhibitor, plasminogen activator inhibitor-1); 7) immunoglobulins; 8) albumin; and 9) regulatory proteins (tissue factor pathway inhibitor, Cl-inhibitor). Alpha-granule membrane specific proteins such as P-selectin (CD62P, PADGEM) fuse with the platelet surface membrane upon α-granule secretion and are conventionally utilized as markers of platelet activation (Figure 3, pg. 20). The lysosomal and dense-granule membrane CD63 similarly becomes fused with the platelet plasma membrane and is a marker of the platelet release reaction. Lysosomes additionally contain a numbers of enzymes that include β-glucuronidase, cathepsins, aryl sulfatase, β-hexosaminidase, β-galactosidase, heparitinase, elastase and collagenase [9]. The elastase and collagenase activities may contribute to vascular damage at the site of platelet activation [36]. Dense-granules are rich in ADP, ATP, GTP, GDP, metal-ions (Mg²⁺, Ca²⁺) and serotonin. Ca²⁺ released from dense bodies, upon
platelet activation, is in high local concentration that may contribute to the platelet activation processes \[9,36\].

### 1.2.6 Platelet membrane procoagulant activity

While the presence of activated platelets is generally accepted to be necessary for in vivo thrombin generation, the exact mechanisms of membrane procoagulant activity continue to be elaborated \[9\]. Platelet activation leads to the exposure of negatively-charged phospholipids, chiefly phosphatidylserine (PS), at the outer membrane leaflet and the shedding of microparticles \[38,39\]. These events are the connection between platelets and coagulation cascade of enzymes that leads to formation of thrombin. The negatively-charged phospholipids provide the surface for assembly of coagulation protein catalytic complexes such as tenase (VIIIa/IXa) and prothrombinase (Xa/Va), whose activity results in Xa and thrombin generation, respectively, and ultimately fibrin formation \[21,39,40\]. Activated platelet membrane procoagulant activity and MP surface procoagulant activity are similar. To date, it is not clear what the relative contributions of platelets and MPs to the coagulation process are \[21,41\]. Activated platelet membrane and MP surface procoagulant activity are similar. This is further discussed in the section about procoagulant activity of MPs (section 1.5.4; pg. 26).

### 1.3 Platelet membrane receptors

There are more than 40 glycoproteins known to be present on the resting platelet surface \[9,42\]. In addition, a number of others glycoproteins such as P-selectin (CD62P) and lysosomal CD63 are expressed upon platelet activation \[9,42\]. In the following text, some of the more important platelet glycoproteins including GPIIb/IIIa will be briefly reviewed.
1.3.1 Integrins and the platelet GPIIb/IIIa (αIIbβ3) receptor

The platelet GPIIb/IIIa receptor belongs to the integrin family of membrane receptors that contain two non-covalently linked α and β subunits (Figure 1). Integrin mediated signals regulate cell-cell and cell-extracellular matrix interactions, both of which are important in a wide variety of biological processes, including morphogenesis, hemostasis and thrombosis, wound healing, immune system function and metastasis. At present, the integrin family is known to contain 17 α and 8 β subunits, resulting in 22 different integrin complexes with distinct ligand-binding profiles [43]. Integrins are expressed constitutively on the surface of all nucleated cells in the body. Integrins mediate both adhesion and bi-directional transmembrane signalling. Integrin function appears to be regulated by conformational structural changes and receptor clustering. “Inside-out” signalling (affinity modulation) results in the switching a resting integrin to a ligand-binding competent receptor. Following ligand binding, the occupancy information is transduced across the plasma membrane in a process termed “outside-in” signalling (avidity modulation), that triggers integrin clustering and attachment to the membrane skeleton [43].

GPIIb/IIIa integrin glycoprotein is a Ca^{2+}-dependent heterodimer consisting of non-covalently linked GPIIb (αIIb) and GPIIIa (β3) subunits (Figure 1). The GPIIb subunit is found only in combination with the β3 subunit, creating the complex that is located exclusively on platelets. The β3 subunit, in contrast, is also the part of vitronectin receptor (αvβ3) that is also found on other cells (endothelial, smooth muscle cells, melanoma cells, osteoclasts or leucocytes) [43]. GPIIb/IIIa is the dominant platelet receptor, with 40,000 to 80,000 receptors present on the surface of a resting platelet. Another 20,000 to 40,000 receptors are found internally, primarily in α granule membranes and the membranes lining the open canalicular system [9,42]. Intracellular GPIIb/IIIa become translocated to the
membrane surface and activated upon platelet activation. The physiological significance of GPIIb/IIIa is best illustrated by the congenital bleeding disorder, Glanzmann’s thrombasthenia (GT), in which identified mutations result in a functional deficiency of the GPIIb/IIIa receptor, resulting in the absence of agonist-induced platelet aggregation [44].

The exact mechanism of GPIIb/IIIa activation remains obscure [20]. Studies have suggested that PKC-dependent signalling events (possibly via cytoplasmic protein plekstrin (P47) phosphorylation) are required to activate GPIIb/IIIa. Lack of “inside-out” signalling that transforms GPIIb/IIIa receptor to its active form and an inability of platelets to aggregate, were correlated with diminished pleckstrin phosphorylation in a recently reported patient [45]. Further, the cytoplasmic domains of GPIIb and GPIIIa are both involved functionally, in activation as both mutations and deletions in these domains give rise to GPIIb/IIIa molecules that are either non-activatable or are permanently activated [46].

In resting platelets, the GPIIb/IIIa receptor binds fibrinogen to only a limited extent during the constitutive transport and incorporation of fibrinogen into α-granules or when fibrinogen is bound to a surface [47,48]. Platelet activation induces conformational changes of GPIIb/IIIa, making it fibrinogen-binding competent [19]. These conformational changes can be detected by the binding of PAC-1, a monoclonal antibody to GPIIb/IIIa that recognizes an activation specific epitope [49]. Upon ligand binding, the GPIIb/IIIa receptor expresses new epitopes not previously available, termed “ligand induced binding sites” (LIBS) [50]. LIBS can be detected with monoclonal antibodies such as PMI.1, specific only for the fibrinogen-bound form of GPIIb/IIIa receptor [51]. LIBS can be located either on the extracellular or on the cytoplasmic parts of the GPIIb/IIIa complex [52]. Ligand-binding competent GPIIb/IIIa receptor sends “outside-in” signals that lead to tyrosine phosphorylation of the cytoplasmic tail of the β3 unit and the clustering of GPIIb/IIIa receptors over the platelet membrane
surface by connecting to the platelet cytoskeleton [53]. Fibrinogen bound to activated GPIIb/IIIa cross-links adjacent activated platelets, resulting in platelet aggregation [13].

Fibrinogen binds to the activated GPIIb/IIIa receptor at three distinct receptor binding sites. The two fibrinogen α-chain RGD sequences bind to β3 (GPIIla) subunit at amino-acids 109-171 (RGDF: 95-97) and 211-222 (RGDS: 572-574) [54,55]. The γ-chain dodecapeptide binding site (HHLGGAKQAGDV; H12), unique to fibrinogen, binds to the αIIb subunit at amino acids 294-314 of GPIIb/IIIa [56,57]. Recently, another binding site for the γ-chain of immobilized fibrinogen has been identified in β3 subunit (amino acids 274-368) [58]. The binding of immobilized fibrinogen to the GPIIb/IIIa through this binding site does not require the activated conformation of the GPIIb/IIIa, as demonstrated by Alemamy et al. [58].
Figure 1 Depiction of GPIIb/IIIa receptor

The platelet GPIIb/IIIa receptor contains αIIb and β3 subunits with extracellular and cytoplasmic domains. Depicted are three putative fibrinogen binding sites: the RGDF and RGDS residing on β3 and the γ-chain H12 on the αIIb subunit. Adapted from Phillips DR, 1988 Blood [59].
1.3.2 Other platelet integrin receptors

GPIa/IIa (α2β1) is another member of the integrin family composed of α2 and β1 subunits. GPIa/IIa was identified as the collagen receptor since platelets that lack GPIa were unresponsive to collagen-induced aggregation [60]. There are approximately 1000 GPIa/IIa receptors on the platelet membrane surface [9]. Collagen binding is Mg²⁺-dependent and platelet activation independent. The signalling events in collagen-activated platelets are not fully understood, although activation of tyrosine kinases and phospholipase Cγ2 (PLC γ2) are involved [25,61,62].

GPIc*IIa (α5β1) fibronectin receptor is a β1 integrin expressed on a wide variety of cells that mediates adhesion to fibronectin. This receptor is competent to mediate adhesion of resting platelets to fibronectin and contributes to platelet adhesion under low flow conditions [63]. There are approximately 1000 copies per platelet [9].

GPIc/IIa (α6β1) laminin receptor is a β1 integrin as well and mediates resting platelet adhesion to subendothelial-exposed laminin with approximately 1000 receptors on the platelet membrane surface [9].

αvβ3 vitronectin receptor is β3 integrin located on the variety of cells including platelets, endothelial cells, smooth muscle cells, melanoma cells, osteoclasts and monocytes. Even though αvβ3 binds vitronectin, fibrinogen, thrombospondin and vWF, there are only 50-100 receptors per platelet [9]. However, being a promiscuous receptor, it mediates platelet adhesion to osteopontin in atherosclerotic plaques, endothelial cells, participates in thrombin generation, endothelial and tumor angiogenesis and cancer dissemination [64-66].
1.3.3 Other platelet receptors

**GPIb/GPIX/V (CD42)**

The glycoprotein GPIb/IX/V complex, a member of the leucine-rich protein family, is the primary platelet receptor for initial platelet surface adhesion under high shear conditions, and binds to the ligand vWF [24]. There are approximately 25,000 GPIb/IX/V receptors per platelet [9]. High shear force is critical for this interaction, as GPIb/IX/V receptor and vWF do not interact and participate in platelet adhesion at low shear forces [10,12]. In resting platelets the GPIb cytoplasmatic domain is connected to platelet cytoskeleton via actin binding protein [67]. GPIb/IX/V binding of multimeric vWF triggers intracellular signalling and activation of the membrane GPIIb/IIIa receptor, inducing platelet aggregation in vivo [10]. GPIb/IX/V is also involved in platelet activation by providing a binding site on the platelet surface for thrombin [68]. The physiological importance of the GPIb/IX/V complex, in platelet adhesion, is best illustrated by the congenital bleeding disorder Bernard-Soulier syndrome (BSS). BSS platelets lack surface expression of GPIb/IX/V complex and are not able to adhere to subendothelium [3].

**P-selectin (CD62P, GMP-140, PADGEM)**

P-selectin is located in platelet α-granule membranes and upon platelet activation is translocated to the platelet membrane surface [37]. There are around 13,000 molecules per platelet. CD62P belongs to the selectin family of adhesive proteins that includes the endothelial leukocyte adhesion receptor E-selectin (ELAM-1) and lymphocyte receptor L-selectin (LAM-1). P-selectin mediates Ca$^{2+}$-dependent platelet-leukocyte interaction through its counter-receptor P-selectin glycoprotein ligand-1 (PSGL-1) [9,36].
GPVI and GPIV (CD36)

Other platelet collagen receptors include GPIV (CD36; also known as GPIIIb)) and GPVI [25,69,70]. GPVI has been identified as a collagen receptor since platelets from GPVI deficient patients and in patients with ITP, characterized by anti-GPVI antibodies, both manifest a lack of collagen interaction and aggregation [25,62,69]. Activation of the GPIIb/IIIa receptor is mediated by both collagen receptors (GPIa/IIa and GPVI) [71-74].

1.4 Platelet ligands

1.4.1 Fibrinogen

Fibrinogen is synthesized by hepatocytes and circulates in plasma. Substantial amounts (70-140 µg/10⁹ platelets [75,76]) are stored in platelet α-granules following uptake of plasma fibrinogen in a process that involves cycling of the GPIIb/IIIa receptor from the membrane surface to internal granules [48,77-79]. Alpha-granule fibrinogen (internal fibrinogen) is absent in patients with afibrinogenemia and in Glanzmann's thrombasthenia type I [80]. Plasma fibrinogen is a dimerized protein that contains three pairs of polypeptide chains Aα, Bβ and γ (Figure 2) [81]. Fibrinogen binds to the activated form of GPIIb/IIIa receptor through two α-chain RGD (α-chain RGDF 95-97 and RGDS 572-574) and a carboxy terminus γ-chain HHLGGAKQAGDV (γ-chain H12; 400-411) sequence [82-85]. At present, the relationship between the α-RGD and γ-chain H12 binding sites remains unclear and binding of these two motifs to the GPIIb/IIIa receptor is mutually exclusive [86,87]. Peptides containing either the γ-chain H12 or RGD sequence and antibodies against either peptide prevent fibrinogen binding to GPIIb/IIIa [84,86,88,89]. The γ-chain H12 has an essential role in platelet aggregation and adhesion demonstrated through studies involving recombinant mutant fibrinogens (rFbg) defective in RGD and γ-chain H12 sites [90,91].
Mutant rFbg with an RGD/RGE substitution site at either α-chain 97 or α-chain 574 (rFbg-D97E; rFbg-D574E) had normal ability to aggregate ADP-stimulated platelets [90] while mutant fibrinogen defective in the γ-chain H12 site (rFbg-γ') was markedly deficient in its aggregating ability [91]. Further evidence comes through the electron microscopic studies of the relationship between the putative fibrinogen binding sites and the GPIIb/IIIa receptor confirming that over-bridging between two GPIIb/IIIa receptors in the process of platelets aggregation is realised through carboxy terminal γ-chain binding sites of fibrinogen molecule [92]. Further, this key role of γ-chain H12 in binding to GPIIb/IIIa complex, platelet aggregation and adhesion, was further demonstrated, by binding studies using peptide and specific monoclonal antibody (4A5), to be located within carboxy terminal AGDV sequence of fibrinogen γ-chain (408-411) [93-97]. These studies demonstrate that RGD sequence is not necessary while the γ-chain H12 is critical for platelet aggregation. Synthetic peptides containing RGD or γ-chain sequences inhibit the binding of fibrinogen to the GPIIb/IIIa. These observations have been exploited to produce therapeutic agents to inhibit platelet thrombus formation [86,88].

Carboxy terminus γ-chain H12, unique to fibrinogen, binds exclusively to platelet αIIb subunit [56,57]. Notably, the αIIb subunit is found only in the platelet GPIIb/IIIa integrin [43]. The RGD binding sequence in β3 subunit however is the common binding site for other adhesive ligands (vitronectin, fibronectin, von Willebrand factor) that bind to the GPIIb/IIIa receptor as well [88]. In addition, many other integrins (αvβ1, αvβ3, αvβ5, α3β1, α5β1, α8β1, αvβ6) bind a series of proteins (collagen, fibrinogen, fibronectin, vitronectin, prothrombin, thrombospondin, disintegrins, vWF) in an RGD-dependent manner [43,98].
Binding of fibrinogen to GPI\(\text{IIb/IIIa}\) induces conformational changes in fibrinogen, termed “receptor-induced binding sites” (RIBS), that can be recognized by specific monoclonal antibodies, such as antibody 9F9 [50].

1.4.2 Fibrin

At sites of vascular injury, fibrinogen is converted by thrombin to fibrin monomer by the cleavage of fibrinopeptides A and B from the amino-terminal ends of the fibrinogen A\(\alpha\) and B\(\beta\) chains (Figure 2). The fibrin monomers then polymerise spontaneously and undergo intermolecular linking to form a stable fibrin clot in the presence of transglutaminase (factor X\(\text{IIIa}\)). Fibrin is the most abundant protein in a thrombus [81]. New platelets are recruited to the site of vascular injury through the adhesion of the GPI\(\text{IIb/IIIa}\) receptor to fibrin mesh, increasing the size of the thrombus [12]. GPI\(\text{IIb/IIIa}\) also participates in clot retraction as demonstrated in studies of patients with Glanzmann’s thrombasthenia and studies with GPI\(\text{IIb/IIIa}\) inhibitors [80,99]. Fibrin is bound primarily through the three recognized fibrinogen binding sites (RGD and H12), but other binding sites may be involved as well since mutant fibrinogen, lacking all three putative binding sites still support fibrin clot retraction [94,96]. The role of the fibrin polymer is to stabilise the thrombus at the site of vascular injury, preventing blood loss.
Figure 2 Schematic diagram of fibrinogen

There are 3 sets of polypeptide chains, two Aα, two Bβ and two γ-chains, containing GPIIb/IIIa binding sites (α-RGDF: 95-97; RGDS: 572-574; γ-chain H12: 400-411). Thrombin cleaves off fibrinopeptide A and B to yield fibrin monomer. Adapted from Rosenfeld JS, 1997 Acta Hematol [24].
1.4.3 Other RGD-containing GPIIb/IIIa binding ligands

Besides fibrinogen, other vascular adhesive ligands including vWF, vitronectin and fibronectin bind to the GPIIb/IIIa receptor through a common RGD recognition specificity, interacting with the GPIIIa subunit of the integrin complex [88,100,101]. Notably, the GPIIIa subunit can be also found in combination with the αv subunit, forming the vitronectin receptor located on cells other than platelets (smooth muscle cells, endothelial cells; see vitronectin receptor pg. 12) [43].

Von Willebrand factor is a multimeric adhesive glycoprotein synthesized by endothelial cells and megakarocytes that is the carrier protein for factor VIII. It is stored in the α-granules of platelets and Weibel-Palade bodies of endothelial cells (EC) and is secreted from EC both luminally and abluminally into the extracellular matrix and plasma. The main platelet-vWF binding receptor is the GPIIb/IX/V complex [18]. Congenital deficiency of vWF, von Willebrand disease, is a bleeding diathesis manifested in part by an inability of platelets to adhere to surfaces [80].

Vitronectin and fibronectin are present in plasma, platelet α-granules and the subendothelial matrix. Even though each binds to its own specific platelet receptor, they are both capable of binding to the GPIIb/IIIa receptor as well, through the common recognition RGD sequence [101]. However, resting platelets do not bind plasma vitronectin or fibronectin. Plasma and α-granular fibronectin appear to be different from fibronectin present in exposed subendothelial matrix. This may be a mechanism for controlling platelet activation through interaction with fluid phase ligands [11]. Vitronectin binding requires prior activation of the vitronectin receptor or of GPIIb/IIIa. The significance of the binding of fibronectin and vitronectin to the GPIIb/IIIa receptor is not clear, but binding of platelets
to immobilized vitronectin and fibronectin in exposed subendothelium may initiate platelet adhesion and activation [17].

1.4.4 Collagen

To date, 19 different types of collagen have been identified [102]. Collagen type I is the most ubiquitous fiber type and is the major constituent of skin, hypodermis, fascia, tendons, ligaments, interstitium, fibrous cartilage, bones and blood vessels wall [102]. Collagen type I is synthesized by fibroblasts and by blood vessel smooth muscle cells and is a heterotrimer comprising two α1(I) and one α2(I) polypeptide chains, each containing around 1400 amino acids (MW~130 Kd). Every third amino acid is glycine, allowing the α-chains to adopt the characteristic collagen triple helix [102]. The ability of collagens to promote platelet adhesion, aggregation and secretion differ among the different types of collagen [70]. Collagen type I, together with II, III and IV, comprise the highly platelet reactive (adhesive) collagens under both static and flow conditions in vitro [25,70,103]. As a major constituent of subendothelial matrix, collagen is considered to be the most thrombogenic material that platelets can encounter in vivo [25,70]. Collagen platelet receptors include GPIa/IIa (α2β1) [60], GPIV (CD36, also known as GPIIIb)) and GPVI [69].
Figure 3 Platelet activation and MP generation

Upon platelet stimulation with thrombin, thrombin receptor activating peptide SFLLRN, collagen, complement or ADP, platelets transform from a resting to an active form. Platelets release α-granule content expressing P-selectin on the membrane surface (CD62P) and releasing fibrinogen. Membrane surface receptor GPIIb/IIIa becomes up-regulated and fibrinogen competent, while the GPIb receptor becomes down-regulated. As a result, platelets aggregate, adhere and generate microparticles. Microparticles retain some of the characteristics of platelet membrane surface. Platelet adhesion to subendothelial matrix, composed of fibrinogen (Fgn), von Willebrand factor (vWF), vitronectin (Vtn), fibronectin (Fn) or collagen (Coll) is mediated by specific platelet membrane receptors.
1.5 Platelet-derived microparticles—background knowledge

Platelet-derived microparticles (MPs) are platelet membrane vesicles with negatively-charged aminophospholipids exposed on the outer membrane leaflet that are generated upon platelet activation. Early observations indicated that in addition to the contribution made by platelets to the coagulation process, a subcellular element, not detected by common methods, was involved [104,105]. This procoagulant activity, found in platelet-poor plasma (PPP) and serum could be eliminated with high-speed centrifugation [104,105]. Later on, using scanning electron microscopy (SEM), Wolf demonstrated numerous tiny membrane fragments in PPP and serum, that he named “platelet dust” [106]. He showed that this “platelet dust” had procoagulant activity similar to activated platelets [106]. Today, it is commonly accepted that MPs arise as a consequence of platelet activation. Because of their minute size (less than 0.8 μm), studies on MPs were hampered until the development of modern flow cytometric methods [107]. While there is growing research interest in MPs, their biological properties, the mechanism of their generation and their role in the circulation remained uncertain.

1.5.1 Clinical aspects of MPs

1.5.1.1 Elevated MP generation

MPs have been hypothesized to be highly procoagulant in the circulation. Platelet microparticulization in the circulation was reported by George et al., in patients undergoing open-heart surgery [108]. Subsequently, atherosclerotic changes in the blood vessel wall were recognized as underlying pathophysiological mechanisms that were associated with platelet activation and microparticle generation [109,110]. Elevated MP levels in the circulation have been observed in a number of cardiovascular disorders involving platelet
activation. These include thrombosis and vessel occlusion, myocardial infarction (MI), acute coronary syndromes (ACS), percutaneous transluminal coronary angioplasty (PTCA), cardiopulmonary bypass (CPB), respiratory distress syndrome (ARDS) and cerebrovascular accidents (CVA) [4,5,111-115]. Elevated MPs have also been observed in a number of clinical conditions characterized by increased platelet activation, thrombosis and thrombocytopenia including idiopathic thrombocytopenic purpura (ITP) [6,116,117], heparin-induced thrombocytopenia (HIT) [118-120], thrombotic thrombocytopenic purpura (TTP) and the hemolytic uremic syndrome (HUS) [7,121]. MPs have also been reported in several conditions characterized by both activated coagulation and fibrinolysis such as disseminated intravascular coagulation (DIC), eclampsia, myeloproliferative disorders and sepsis [8,122]. Finally, increased thrombotic risk in paroxysmal nocturnal hemoglobinuria (PNH), a clonal stem-cell disorder in which blood cells are overly sensitive to complement initiated lysis due to the lack of cell-surface complement inhibitors CD59 and CD55, correlates with high levels of circulating procoagulant MPs [123,124]. While MPs are generally accepted to play a physiologic procoagulant role in the circulation, it is important to note that MP also express potential anticoagulant properties due to the ability to provide the surface for the activation of protein C and the inactivation of factor Va [125].

1.5.1.2 Deficient MP generation

Further evidence of platelet MP possible role in circulation arises from two congenital bleeding disorders characterized by impaired ability to generate MP upon platelet stimulation: Glanzmann's thrombasthenia type I and Scott syndrome.
1.5.1.2.1 **Glanzmann's thrombasthenia type I**

The congenital bleeding diathesis, Glanzmann's thrombasthenia type I, is characterized by the inability of platelets to aggregate, due to a deficiency of the GPIIb/IIIa receptor [44]. Besides an inability to aggregate, platelets deficient in GPIIb/IIIa also do not shed MPs upon thrombin stimulation [126]. The impaired ability of platelets from Glanzmann's thrombasthenia type I to generate MPs is related to the absence of the GPIIb/IIIa receptor, and is direct evidence that the platelet GPIIb/IIIa receptor and its ligands, potentially fibrinogen, play a central role in the process of MP generation [89,126].

1.5.1.2.2 **Scott syndrome**

The physiologic credibility of MP formation as an important event in platelet coagulant activity is supported by another bleeding diathesis with a defect in platelet MP generation [21,127,128]. Patients with Scott syndrome have a genetic defect of an enzyme (phospholipid scramblase) that is involved in platelet membrane phospholipid randomization, leading to procoagulant surface exposure [128]. Upon otherwise normal platelet activation, mutations of their scramblase enzyme system result in an inability to expose negatively-charged PS on the outer membrane leaflet upon platelet activation, reduced ability to promote both tenase and prothrombinase activity in response to all agonists and impaired capacity to shed membrane-derived MPs [21,38,39,128-130]. Scott syndrome may be manifested by significant bleeding complications [128,130].
1.5.2 Characterization of MPs

1.5.2.1 MP size

Flow cytometry is the most widely utilized method of MP detection. MPs are accepted to be smaller than platelets, ranging in size from 0.2-0.8 μm and expressing the platelet membrane surface specific receptors, GPIIb/IIIa and GPIb [107]. However, when assessed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) MPs comprise a much more heterogeneous population whose size is often smaller than 0.1 μm [40,131-133]. Sandberg et al., using SEM, reported that collagen-stimulated platelets generated two populations of MPs: 0.08–0.2 μm and 0.4-0.6 μm [131]. The size difference noted by these two methods is presumably due to the inability of flow cytometers to resolve particles size smaller than 0.1 μm. Further, flow cytometry is not capable of discriminating between very small platelets and MPs and therefore the two populations may overlap [134]. Microparticles separated from stimulated platelets by filtration [135], differential centrifugation [136] or ultracentrifugation (over 100,000 xg forces) [137,138] have been assessed by SEM and found to average 0.1-0.2 μm in size and even smaller.

1.5.2.2 MP membrane composition

The membrane composition of MPs reflects the content of activated platelet membranes. The platelet receptors GPIIb/IIIa and GPIb, critical for platelet aggregation and adhesion, are routinely identified on the surface of MPs by specific monoclonal antibodies [21,34,40,107,108]. MP membranes also express P-selectin [108,139], GPIIa (β1 integrin; a component of collagen receptor (α2β1), fibronectin (α5β1) and laminin receptor (α6β1)) [34,140], GPIV [34], CD109 [141] and CD9 [142,143]. MP membranes are rich in binding sites for activated coagulation factors Va [40,127] and VIIIa [144]. Platelets labelled with
biotin or with the lipophilic dye (PKH2) prior to activation shed biotinylated/PKH2 labelled MPs [145,146]. Since the platelet membrane is rich in different glycoproteins, it is possible that MPs have on their membrane other platelet receptors that have yet to be identified. As determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), MPs also carry on their surface other substances also found in platelets a-granules including thrombospondin, platelet factor 4, β-thromboglobulin and fibrinogen [34]. Fox et al., have shown that the shedding of MPs from platelet membrane surface is correlated with calpain-mediated cytoskeleton proteolysis and that actin, actin binding protein, talin, and myosin heavy chain are MP components [34]. Others have identified the presence of calpain in MPs [127,134,147].

1.5.3 Generation of MPs

It is generally accepted that platelet activation is required for MP generation (Figure 3, pg 20). George et al., found that the concentration of MPs in serum was 10-fold than that in plasma, implying that MPs are produced during blood clotting [148]. Indeed, MPs are generated in vivo and have been observed in a number of clinical conditions characterized by platelet activation and arterial and venous thrombosis (see clinical aspects of MPs, section 1.5.1.1, pg. 21). In addition to the up-regulation of GPIIb/IIIa, down-regulation of GPIb and P-selectin expression by platelets, MPs are considered as a marker of platelet activation easily assessed by flow cytometry [149].

MP generation in vitro is dependent on the type of the agonist used [127]. Physiological platelet agonists that generate MPs include thrombin (together with PAR1 thrombin receptor activating peptide, SFLLRN), collagen, complement terminal attack complex (C5b-9) and ADP [21,40]. Calcium-ionophore A23187 and phorbol myristate acetate (PMA) are non-
physiological agonists able to do the same [126,138]. Epinephrine is weak agonist capable of activating platelets but not able to induce MPs. The degree of platelet microparticulization in vitro depends on the type of agonist used: the strongest is calcium-ionophore A23187, then the combination of thrombin and collagen, followed by the complement attack complex C5b-9 [127]. Some CD9 monoclonal antibodies have been reported to induce platelet microparticulization through activation of the complement system [143,150,151]. Complement platelet activation is believed to be a major pathway leading to MP formation in some clinical disorders (ITP, TTP, PNH, DIC) [152,153]. Freeze/thaw cycles have been shown to generate MPs-like structures but their relationship to physiologically induced MPs is unknown [154]. Use of prosthetic devices, hemodialyzers and coronary by-pass machines in modern medicine has been hampered by thrombogenicity and platelet activation, with shedding of MPs [155,156]. MP generation may be triggered in arterial circulation by high shear forces such as encountered in severe atherosclerotic arteries and in simulated vascular models in vitro [112,157]. MPs can arise in stored platelet concentrates and cryoprecipitates as part of reported "platelet storage lesion", a recognized problem in transfusion medicine in which the combination of platelet contact with bag wall or/and cold activated platelets, induces platelets to change their shape, secrete internal contents and shed MPs [158]. There is an increased risk of thrombosis if such preparations are transfused and this risk correlates to MP levels [159,160].

1.5.4 MP procoagulant activity

A main functional property of MPs is generally believed to be in their procoagulant activity. It has been reported that most of the platelet-derived catalytic surface for coagulation cofactors Va [21] and VIIIa are located on MP membrane surface [144]. In
contrast to platelets, MPs express a steady increase in high affinity receptors for factors Va and VIIIa while the membrane of activated platelets becomes depleted of binding sites for these enzymes within 30 min of activation [144]. Further, high affinity factor IXa binding sites and Xa activity is concentrated on MP surfaces, compared to activated platelets [161,162].

Plasma membrane phospholipids (PL) are normally distributed asymmetrically so that phosphatidylcholine (PC) is primarily located in the outer leaflet while phosphatidylethanolamine (PE) and negatively-charged phosphatidylserine (PS) are found in the inner leaflet. Asymmetry is maintained by a flip-flop system of enzymes (translocase, floppase and scramblase). Energy (ATP)-dependent translocase and floppase enzymes maintain asymmetry, while the Ca^{2+}-dependent scramblase, activated by cell activation and a rise in Ca^{2+}-ions, allows for randomization of phospholipids [163,164]. Imbalance of phospholipid transport, with outer exposure of inner leaflet negatively-charged PS results in cell blebbing and subsequent shape changes, that are compensated by the release of MPs. Simultaneously, a rise in Ca^{2+} activates calpain and brings about the cytoskeletal reorganisation with MP shedding (Figure 4) [127,134]. Extensive work of Bevers, Zwaal, Comfurius and others, linked exposure of negatively-charged aminophospholipids (chiefly PS) at the outer leaflet of platelets to the coagulant ability that is identified in the older literature as platelet factor 3 activity (PF3) [163,164].

Exposure of negatively-charged PS on the MP membranes provides a catalytic surface for the activation of coagulation factor X by activated factors VIIIa and IXa (so called “tenase” complex) and of factor II (prothrombin) by activated factors Va and Xa (prothrombinase complex), leading to thrombin formation (Figure 4 and 5) [38,165,166]. The physiological significance of these events is best illustrated by the congenital defect in phospholipids
scramblase, Scott syndrome, which is characterized by serious bleeding complications and lack of MP generation, inability to expose negatively-charged phospholipids, and reduced tenase and prothrombinase coagulation complex assembly (see Scott syndrome, section 1.5.1.2.2, pg. 23) [21,38,39,128-130]. The use in flow cytometry of annexin V, a placental protein with high affinity and specificity for PS, is a rapid method to assess procoagulant activity in platelets/MPs and the loss of phospholipid asymmetry in cell membranes [39]. It appears also that binding of annexin V prevents the binding of factor Xa to activated platelets [167].
Asymmetry of phospholipid distribution in resting platelets is maintained by translocase and floppase enzymes. Increase in cytosolic calcium leads to activation of phospholipid scramblase that results in the exposure of negatively-charged aminophospholipid (PS) and in calpain-mediated platelet cytoskeletal reorganization. Exposure of PS at the outer leaflet of platelet membrane and calpain activation is followed by membrane blebbing and shedding of membrane vesicles rich in binding sites for tenase and prothrombinase complex assembly (IXa/VIIa and Va/Xa, respectively) [164].
Although the procoagulant activity of platelet-derived microparticles has been questioned, and assigned to intact activated platelets [41], there is an increasing body of evidence in support of procoagulant role of MPs. The observation of MPs in a growing number of clinical conditions suggests a possible procoagulant in vivo role for these particles (see clinical aspects of MPs, section 1.5.1, pg. 21) [4-8,108,111-117,120,122,123].

It is evident that the negatively-charged phospholipid surface of activated platelets and of MPs, dramatically enhances the activation of coagulation proteins and thrombin formation, thereby leading to fibrin clot retraction and the stabilization of growing thrombi on the platelet aggregate nidus [163]. It is likely that during platelet activation and coagulation, both platelets and MPs can provide a catalytic surface for coagulation complex assembly. However, there is evidence that MPs provide this surface for concentrated and sustained coagulation enzyme assembly, implying long-lasting procoagulant activity compared to platelets, and the potential for vascular dissemination from the original site of generation [21,40,144]. Studies have shown that the densities of amino-phospholipids on MPs are higher than are those on remnant platelets [39,166]. Additionally, MPs have a larger surface-to-volume ratio than do platelets [21,40,144]. Finally, Scott syndrome platelets, deficient in both MP generation and PS exposure, may underscore the importance of the procoagulant role of MPs in the circulation (see Scott syndrome, section 1.5.1.2.2, pg. 23) [21,128].
Figure 5 Coagulation cascade and the role of MPs

Coagulation cascade: MPs provide a catalytic surface for tenase (VIIIa/IXa) and prothrombinase (Xa/Va) complex assembly. Thrombin, once generated, cleaves fibrinogen into fibrin and activates other platelets and coagulation factors (XIII, XI, VIII and V).

Abbreviations: calcium ($\text{Ca}^{2+}$); phospholipids (PL); microparticles (MPs); tissue factor (TF).
1.5.5 MP adhesive properties and ligand binding

Very little is known about ability of MPs to adhere to the vascular ligands that support the adhesion of circulating platelets (fibrinogen, fibrin, collagen, vitronectin, fibronectin or vWF). Due to their low buoyant density, MPs can travel throughout the blood stream, and can therefore provide a procoagulant surface for coagulation cascade enzyme assembly at sites that are distant from the site of initial MP formation. But whether they have ability to anchor as platelets do, both locally and at distance from the site of platelet activation, is largely unknown. Studies that would provide insight into the ability of MPs to adhere have been sporadic and contradictory [64,136,154,168-170]. The difference among these studies may arise partly from the difference in the technical preparation of MPs. Further, there are few studies that have addressed whether the adherent properties of MP membrane receptors are similar to those of platelets [170]. It has been reported that the GPIIb/IIIa receptors of MPs have different properties compared to those of platelets [170]. It is believed that the difference arises from the disconnection of GPIIb/IIIa from platelet cytoskeleton, during the last steps of platelet activation and MP formation. It is presumed that this disconnection from the cytoskeleton affects the function of GPIIb/IIIa in binding fibrinogen [170]. In contrast, Holme et al. have reported that MPs are able to bind both fluid phase and immobilized fibrinogen implying that the MP GPIIb/IIIa receptor is functional [168]. In an experimental model of thrombosis in which platelets were stimulated with thrombin or collagen, GPIIb/IIIa positive vesicles smaller than 1 μm were found to bind fibrin as assessed by both immunofluorescence and scanning immunoelectron microscopy [136]. This finding was supported by an in vitro ELISA assay, in which MPs bound most avidly to surface-bound fibrin, and less so to fibrinogen, collagen or vWF [136]. The authors also reported that MP adhesion was agonist dependent. Calcium-ionophore A23187-induced MPs were more
adhesive than were those induced by thrombin or collagen [136]. Freeze/thaw generated MPs were demonstrated to adhere to endothelial cells under the conditions of increasing shear rate and were also shown to activate platelets [154]. However, whether MPs generated by freeze/thaw method are comparable to those generated by more physiological methods is not certain [154]. Another study found that calcium-ionophore A23187-induced MPs were non-adherent to stimulated and non-stimulated human umbilical vein endothelial cells (HUVECs), but were adherent to subendothelial matrix fibrinogen, fibronectin and collagen, with minimal binding to vitronectin and vWF [169]. The adhesion of MPs to immobilized fibrinogen and fibronectin was GPIIb/IIIa dependent and was inhibited up to 90% by Integrilin and ReoPro, novel GPIIb/IIIa blockers [169]. It is important to note that there are few reports that identify the interaction of MPs with other cells (platelets, endothelial and leukocytes (monocytes)) indicating potential stimulatory role of MPs in these interactions [64,168,171,172].

1.5.6 Mechanism of MP formation

The mechanism of MP formation remains incompletely understood. We and others have shown that MP generation is Ca^{2+}-ion and agonist dependent [133,136]. The involvement of protein kinases and calpain in MP formation has also been suggested [34,38,40,127,133,134,147].

It has been shown that the platelet GPIIb/IIIa receptor plays a role in MP generation [126]. MP formation was inhibited by the tetrapeptide adhesion ligand, RGDS or by monoclonal antibodies to the GPIIb/IIIa receptor [126]. Furthermore, Glanzmann's thrombasthenia type I platelets lacking the GPIIb/IIIa receptor, were markedly impaired in their ability to generate MPs upon stimulation with strong agonists (PMA, thrombin, SFLLRN or collagen) [126]. In
addition, GPIIb/IIIa blockade with monoclonal antibody 7E3 (which prevents platelet fibrinogen binding and aggregation) resulted in both reduced thrombin generation and inhibition of MP formation [89]. The role of GPIIb/IIIa in MP generation was confirmed in a study that used SFLLRN-stimulated Glanzmann's thrombasthenic platelets [173]. Curiously, they found that platelets from Glanzmann's patients stimulated with CD9 monoclonal antibody (that activates platelet through activation of complement system; clone FN52) were capable of generating MPs [173]. Regardless of the agonist used, MPs expressed negatively-charged aminophospholipids (PS) [173]. Thus there may be different mechanisms for MP generation - one GPIIb/IIIa-dependent and the other GPIIb/IIIa-independent - with the exposure of negatively-charged aminophospholipids (PS) common for both [173]. Just recently, a link between the GPIIb/IIIa receptor, MPs and PS has been established. It has been demonstrated that the GPIIb/IIIa inhibitory drugs ReoPro and Integrilin, that prevent fibrinogen binding and platelet aggregation, also prevent aminophospholipid (PS) exposure on the outer surface of platelets as assessed by annexin V binding [174]. The mechanism underlying these events is unknown. Thus the relative roles and requirements of GPIIb/IIIa fibrinogen binding and of two RGD and γ-chain H12 binding sites in MP generation, or other adhesive ligands that bind GPIIb/IIIa receptor with RGD specificity (fibronectin, vitronectin and vWF) remain unclear.

1.6 Statement of the Problem (Study rationale)

Platelet-derived microparticles are highly procoagulant membrane vesicles generated during physiological platelet activation. Due to their small size, relatively little is known about them. Specifically, the biologic mechanism of MP generation, and their adhesive properties relative to those of platelets are not well understood.
1.7 Hypothesis

We hypothesize that MP formation requires fibrinogen, its peptides (γ-chain dodecapeptide, RGDS) and platelet GPIIb/IIIa receptors. We hypothesize further that the adhesive properties of MPs resemble those of platelets.

1.8 Experimental objectives

The purpose of this study was:

1. To determine and characterize the roles of fibrinogen, its adhesive peptides (RGD and γ-chain H12), other RGD-containing ligands (vitronectin, fibronectin and von Willebrand factor) and of the GPIIb/IIIa receptor in MP generation, using flow cytometry.

2. To determine whether the adhesion of MPs to protein-coated microspheres is similar to that of platelets, and to characterize the mechanism by which MPs adhere to fibrinogen, fibrin and collagen type I using a unique microsphere based adhesion assay, flow cytometry, SEM and immunogold SEM.

The long term goal of this study is to understand better the role of MPs in hemostasis and coagulation and to gain insight into how these processes may be controlled to prevent pathological thrombosis.
“Generation of platelet microparticles requires fibrinogen and GPIIb/IIIa: specific targets for anti-platelets drugs and RGD paradigm”

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Part of this work has been presented at the: Annual Meeting of the American Society of Haematology, New Orleans, Dec 1999; Hematology days, Toronto Jan 2000; Heart and Stroke/Richard Lewar Centre of Excellence Scientific Day, Toronto May 2000

“Fibrinogen is an essential requirement for platelet microparticle formation”

An abridged version of this chapter will be submitted in manuscript form to the journal of the American Society of Hematology “Blood”.
Chapter 2 The role of fibrinogen in MP formation

2.1 Abstract

Platelet activation is characterized by several phenomena, one of which is the budding off or vesiculation of membrane surface procoagulant microparticles (MPs). The generation of these highly procoagulant, activation-dependent MPs occurs in the presence of cations and requires the β3 platelet integrin GPIIb/IIIa. The physiology of MP formation is otherwise poorly understood. We have characterized the roles of fibrinogen, its GPIIb/IIIa binding peptides and of inhibitors to GPIIb/IIIa in MP generation, using dual-label flow cytometry. Platelet-rich plasma (PRP) and washed platelets (WP), with or without added fibrinogen, were stimulated by thrombin or the thrombin receptor activating peptide (SFLLRN). Dramatic thrombin-induced WP vesiculation occurred upon addition of fibrinogen, in a cation and fibrinogen concentration dependent manner at 50% optimum dose of purified fibrinogen at OD50=12.5±2.78 μg/ml and plasma fibrinogen at OD50=11.3±4.5 μg/ml. MP formation reached plateau at 30 μg/ml of added fibrinogen. SFLLRN-generated MPs in WP with added purified fibrinogen (OD50=48.3±11.7 μg/ml) or plasma fibrinogen (OD50=58.3±21.8 μg/ml) occurred in a similar manner, reaching plateau at 100 μg/ml. Polyclonal affinity-purified anti-fibrinogen antibodies fully inhibited MP formation of WP, to the level of EDTA control in a concentration dependent manner (IC50=5±0.6 μg/ml). The fibrinogen binding peptides α-chain RGDS and γ-chain H12 fully inhibited MP formation in WP and PRP at near identical molar concentrations (IC50:~70 μM). Pharmacological agents and monoclonal antibodies that inhibit fibrinogen binding to GPIIb/IIIa, platelet adhesion and aggregation including ReoPro (abciximab, 7E3) (IC50=1.5 μg/ml), monoclonal antibodies
directed to GPIIb (clone P2) (IC50=5-10 μg/ml) and GPIIIa (clone SZ21) (IC50=5-10 μg/ml), and the peptidomimetic, Eptifibatide (Integrilin) (IC50=0.1 μg/ml), fully blocked MP formation in a dose-dependent manner. These data demonstrate that the binding of fibrinogen, but not of other RGD ligands (vitronectin, fibronectin, vWF), to GPIIb/IIIa, through either or both the α and γ-chain binding sites, is required for thrombin-induced human platelet vesiculation. Further, this is the first description of the role of the GPIIb binding γ-chain H12 in the generation of MPs. Other studies have indicated that the γ-chain H12 is more important physiologically for platelet adhesion and aggregation than is the α-chain RGD. Further research is necessary to elucidate whether a platelet specific, fibrinogen γ-chain H12 sequence, plays a more important role in MP formation as well.
2.2 Introduction

Platelets are anucleated blood cells that in synergy with vasculature and coagulation proteins provide a primary hemostatic plug at the site of vascular injury. Platelets circulate in resting condition until they encounter adhesive proteins at sites of exposed subendothelial matrix of an injured blood vessel or come in contact with physiological agonist such as thrombin, collagen, ADP, epinephrine or the complement attack complex C5b-9 [1]. This contact leads to platelet activation manifested by shape change, an ability to adhere, aggregate, release intracellular granules contents and elaborate procoagulant membrane activity, including the shedding of membrane vesicles rich in coagulation factor binding sites and procoagulant activity [21]. Beside platelets, other blood cells shed vesicles as a result of cell activation and death or during pathological conditions. Monocytes, red blood cells and endothelial cells all vesiculate and their vesicles are considered to be procoagulant and able to transduce hemostatic or inflammatory signals as well [8,114,175]. However, it has been reported that 75%-90% of all circulating microvesicles, when measured in human blood, originate from platelets [8,114,175].

Platelet-derived microparticles (MP) are membrane vesicles with surface-exposed negatively-charged amino-phospholipids, glycoproteins and glycolipids, generated upon platelet activation with the physiologically relevant agonists thrombin, collagen, ADP or complement attack complex C5b-9 [21,39]. MP membrane composition is similar to that of platelets. MPs express GPIIb/IIIa, GPIb, CD62P (P-selectin), CD109 and α1 integrins [108,139-141] amongst other platelet glycoproteins. MPs also provide a catalytic surface for coagulation cascade tenase (VIIIa and IXa) and prothrombinase (Xa and Va) enzymes complex assembly, leading to thrombin generation and fibrin formation [21,40]. In addition, MPs provide a surface for anticoagulant activity including protein C activation and
inactivation of factor Va [125]. MPs are traditionally identified by flow cytometry as being smaller than platelets and ranging in size between 0.2-0.8 μm. However, they can also be observed by electron microscopy as a very heterogeneous sized populations of particles ranging in size down to less than 0.1 μm [131]. Glanzmann's thrombasthenia type I, that lacks platelet GPIIb/IIIa and Scott syndrome, that lacks phospholipid randomization ability, are inherited bleeding disorders with impaired ability to generate normal levels of microparticles [21,44,126,128,129]. Both conditions are also characterized by the reduced ability of platelets to generate thrombin and may be clinically manifested by serious bleeding episodes [89,166].

Although MPs are generally accepted to be highly procoagulant and are observed in the circulation in a wide range of clinical thrombocytopenic (ITP, TTP, HIT) and arterial and venous prothrombotic conditions (MI, ACS, VTE, CVA), their mechanism(s) of generation, physical and biological properties, and role and contribution to hemostasis are incompletely understood [6,7,110,114,116,117,120,122,176]. Previous work indicated that the platelet β3 integrin GPIIb/IIIa played an essential role in the mechanism of MP generation and suggested a potential role for fibrinogen or other RGD-containing GPIIb/IIIa binding ligands (vitronectin, fibronectin and vWf) in their formation [126].

Fibrinogen is a dimerized ligand containing three pairs of polypeptide chains: Aα, Bβ and γ [81]. It binds to the activated form of the GPIIb/IIIa receptor through two α-chain RGD (RGDF at 95-97 and RGDS at 572-574) and carboxy terminus γ-chain HHGGAKQAGDV (γ-chain H12 at 400-411) sequences [82-85]. At present, the relative relationship between α-RGD and γ-H12 binding sites for fibrinogen adhesion to platelets remains unclear. Peptides containing either the γ-chain H12 or RGD sequence or antibodies against either peptide,
inhibit fibrinogen binding to GPIIb/IIIa [84,86,88,89]. γ-chain H12 and RGD binding to the GPIIb/IIIa receptor has been shown to be mutually exclusive [86]. It has been demonstrated that binding of one peptide excludes simultaneous binding of the other peptide by presumed induction of a conformational change that renders the other peptide binding site of GPIIb/IIIa unavailable [86,87,177]. Studies with mutant fibrinogens defective in either RGD or H12 identified the carboxy terminus of fibrinogen γ-chain as directly responsible for platelet aggregation and adhesion [90,91]. Unlike fibrinogen's unique γ-chain H12 that specifically binds to the GPIIb subunit of GPIIb/IIIa, supporting platelet aggregation and adhesion, the RGD sequence in fibrinogen is a peptide sequence found in a number of vascular ligands and is a binding motif for a large number of integrin receptors located on different cells [43,57]. However, most of the novel pharmacological GPIIb/IIIa blocking agents generated to date have been created by exploiting the ability of the RGD sequence to block fibrinogen binding to the platelet glycoprotein GPIIb/IIIa receptor [178]. Whether this lack of ligand and receptor specificity carries a potential danger when these anti-platelet agents are used remains to be determined [179].

In this study, we characterize the role of the dominant platelet GPIIb/IIIa binding ligand fibrinogen, the fibrinogen peptides RGD and γ-chain H12, monoclonal antibodies to GPIIb and GPIIIa subunit and of the anti-GPIIb/IIIa pharmaceutical agents ReoPro and Integrilin in the process of platelet generation of microparticles in a physiologic milieu of platelet-rich plasma (PRP) and washed platelets (WP). Platelets were stimulated with the physiological agonist human α-thrombin or the PAR1 thrombin receptor activating peptide SFLLRN and assessed with dual-labelled flow cytometric methods for the evidence of MP generation. We demonstrate that fibrinogen is the only one of the GPIIb/IIIa, RGD-containing binding ligands compared with von Willebrand factor, vitronectin and fibronectin, that supports
platelet microparticle generation. Further, we demonstrate that both fibrinogen peptides, α-chain RGDS and γ-chain H12, play a role in the mechanism of MP formation. Finally, by inhibiting MP generation, the GPIIb/IIIa blockers ReoPro and Integrilin will eliminate the catalytic contribution of MPs to thrombin generation. Thus, ReoPro and Integrilin function as anti-coagulants and not just anti-aggregating agents.

2.3 Methods and materials

2.3.1 Materials

Bovine serum albumin (BSA), human α-thrombin, fibrinogen γ-chain peptide 400-411 H12 (His-His-Le-Gly-Gly-Ala-Lys-Gln-Ala-Lly-Asp-Val), EDTA, prostaglandin E1 (PGE1) and apyrase grade VII were from Sigma Chemical Co. (St. Louis, MO); electron microscope grade paraformaldehyde was from Polysciences Inc. (Warrington, PA); GPIIb/IIIa binding tetrapeptide RGDS (Arg-Gly-Asp-Ser), and inactive control peptide GRADSP (Gly-Arg-Ala-Asp-Ser-Pro), were from Calbiochem (La Jolla, CA.); purified human fibrinogen (high grade) was from American Diagnostica Inc. (Greenwich, CT). The purity of this fibrinogen was confirmed by SDS-PAGE; normal pooled human standard plasma kept at -70°C (Cryocheck normal) was from Precision Biological (Canada); standard human plasma fibrinogen and purified fibrinogen concentrations were determined by standard Clauss fibrinogen methodology [180] with STA-fibrinogen 5 reagent (product code 0674; Diagnostica Stago, France) using ST4 automated clot detection instrument (Diagnostica Stago, France); PAR1 thrombin receptor activating peptide SFLLRN (Ser-Phe-Leu-Leu-Arg-Asn) was synthesized by the University of Toronto Peptide Synthesis Centre. Integrilin (Eptifibatide), the KGD (Lys-Gly-Asp) containing hepta-cyclo-peptide that prevents fibrinogen binding to GPIIb/IIIa
receptor and that blocks platelet aggregation was kindly donated by COR Therapeutics (South San Francisco, CA).

2.3.2 Antibodies

Fluorescein isothiocyanate (FITC)-labelled monoclonal antibody against platelet GPIb (CD42b) was from Serotec, (Oxford, U.K.); R-phycoerythrin (PE)-labelled monoclonal antibody to P-selectin (CD62P) was from Monosan, (Netherlands); monoclonal antibodies against GPIIIa (clone SZ21), GPIIb (clone P2) that inhibit binding of fibrinogen to platelet GPIIb/IIIa and prevent platelet aggregation were from Immunotech, (Marseille, France); sheep polyclonal affinity-purified human anti-fibrinogen antibody was from Cedarlane Lab. Ltd, (Ontario, Canada). ReoPro (abciximab, chimeric Fab 7E3 monoclonal antibody) that inhibits binding of fibrinogen to GPIIb/IIIa and platelet aggregation was kindly donated by Centocor (Malvern, Pa). An irrelevant monoclonal antibody of the same isotype (IgG1) against human CD4 from Serotec (Oxford, UK) and CD3 polyclonal antibody from DAKO Corporaton (Carpinteria, CA, USA) were used as controls.

2.3.3 Preparation of platelet-rich plasma and washed platelets

Whole blood was collected without tourniquet from healthy donors (who were not taking medications for the last two weeks) after giving signed informed consent, into 5 U/ml of heparin or 0.38% sodium citrate (final concentration) pre-loaded syringes, after discarding the first 2 ml [181]. Heparin was used as anticoagulant for the experiments with platelet-rich plasma (PRP). Whole blood was centrifuged at 150 xg for 5-10 min at 22 °C to prepare PRP (platelet count was 3-3.5 x10^9/ml) [181]. Washed platelets (WP) were prepared from citrated
PRP (0.38% sodium citrate final) in a three step washing procedure at room temperature [182]. Platelets were washed and resuspended in platelet medium that contained 137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO3, 0.4 mM NaH2PO4, 1 mM MgCl2, 5.5 mM glucose and 0.35 % albumin. The first wash was at pH 6.4 and included PGE1 (1 µM final) and sodium citrate (0.38% final). The second and third washes were at pH 7.4. The final platelet medium was recalcified with 2 mM CaCl2, contained apyrase (0.04 U/ml final) and was brought to a final platelet count of 3-3.5 x 10^8/ml for all experiments.

2.3.4 Generation of platelet MPs in vitro

MPs were generated from PRP or WP upon stimulation with the PAR1 thrombin receptor activating peptide SFLLRN (SF; 100 µM) at 37°C. In addition, MPs were generated from WP upon stimulation with 0.25 U/ml of the human α-thrombin at 22°C (no difference was seen in thrombin-induced MP generation at 37°C). Samples were incubated for 20 min with an initial gentle mix by flicking the eppendorf (10 times), prior to analysis by flow cytometry (see below, section 2.3.6).

2.3.5 Assessment of the roles of fibrinogen, the fibrinogen binding peptides (RGDS and H12) and of the platelet GPIIb/IIIa receptor in MP generation

All antibody, ligand and peptide experiments were performed in PRP or WP. Briefly, 50 µl of WP or PRP were incubated with or without human α-thrombin (0.25 U/ml) or SFLLRN (100 µM), for 20 min with or without increasing concentrations of purified human fibrinogen or normal pooled human plasma at known fibrinogen concentration, with an initial gentle mix (see previous section 2.3.4). All experiments were controlled for, in parallel, with
EDTA (10 mM). Inhibition studies employed increasing concentration of ReoPro, Integrilin, anti-GPIIb monoclonal antibody (clone P2), anti-GPIIIa monoclonal antibody (clone SZ21), fibrinogen peptides RGDS and γ-chain H12 and polyclonal affinity-purified anti-fibrinogen antibody, added prior to platelet activation. Then, 5 µl of WP or PRP were diluted in 50 µl of Hepes-Tyrodus buffer (HTB; containing 137 mM NaCl, 3.7 mM KCl, 16 mM NaHCO3, 5 mM MgCl2, 3.5 mM Hepes, 5.5 mM glucose, 0.2% bovine albumin pH 7.4) and incubated with saturating concentrations of FITC-labelled anti-GPIIb monoclonal antibody and PE-labelled P-selectin monoclonal antibody for 20 min, at room temperature. After dilution and fixation with 1 % paraformaldehyde, samples were analyzed by flow cytometry.

2.3.6 Flow cytometry

Samples were analyzed on a Beckton Dickinson FACScan flow cytometer (Mountain View, CA) with CELLQuest software, formatted for two-colour analysis with light scatter and fluorescence channels set on logarithmic gain. All platelet specific events, including whole platelets and microparticles, were identified as GPIb positive fluorescent events to the right of background irrelevant monoclonal antibody FITC signal. MPs were identified and analyzed by creating multi-gate strategy. MPs were GPIb positive and to the left of whole platelets by forward scatter analysis, by definition smaller than intact platelets. Gates for single intact platelets were distinguished from microparticles by forward scatter size analysis in relationship to size beads (0.1-0.8 µm; Beckman Coulter, CA). Ten thousand positive platelet specific events were analyzed and mean fluorescence units per platelet and microparticle, was quantitated. Microparticles were expressed as percentage of total platelet
specific events (MP%). Platelet activation status was assessed with P-selectin expression using phycoerythrin (PE)-conjugated monoclonal antibody.

2.3.7 Statistics

Results are expressed as mean values of up to 5 experiments (if otherwise not stated) ± standard error of the mean (SEM). An independent Student’s t-test evaluated whether two means were significantly different at the p<0.05 probability level. The means of three or more groups were compared by one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison post test to determine which groups were significantly different from each other.
2.4 Results

2.4.1 The role of fibrinogen in washed platelet MP formation

It has been previously shown that MP generation is agonist and metal-ion dependent, that protein kinases and calpain appeared to be involved and that the platelet β3 integrin GPIIb/IIa receptor plays a key role [40, 126, 127, 133]. The requirement of GPIIb/IIa in MP formation suggested that all or some of the GPIIb/IIa binding ligands (RGD-containing fibrinogen, vitronectin, fibronectin, von Willebrand factor) could be involved [89, 126]. Since fibrinogen is the dominant αIIbβ3 ligand in plasma, we probed the role of fibrinogen alone in MP formation by adding purified fibrinogen (as the source of exogenous fibrinogen) to platelets washed free of other plasma proteins.

MP generation in washed platelets stimulated with human α-thrombin or SFLLRN was dramatically enhanced by the addition of exogenous purified fibrinogen and this enhancement occurred in a cation-dependent manner (Figures 6a and b: SFLLRN-stimulated platelets generation of MP by flow cytometry). Exogenously added human fibrinogen was free of contaminating proteins as assessed by SDS-PAGE, with one single band (340 kDa) observed under non-reduced conditions and three bands under reduced conditions, corresponding to the α (63 kDa), β (56 kDa) and γ (47 kDa) polypeptide fibrinogen chains [183]. There was no contamination with fibronectin, a common contaminant of fibrinogen preparations, since there was no band corresponding to 220 kDa, under reduced conditions (Figure 7). As shown on Figure 8a, resting washed platelets (MP % = 2.9 ± 0.2; n = 13) stimulated with SFLLRN, generated 11.1 ± 0.3 (n = 13) microparticles while the addition of 100 μg/ml of human purified fibrinogen increased MP generation approximately 3-4 fold to 33.8 % ± 1.9; (n = 12; p < 0.001 versus WP without fibrinogen, by ANOVA, see statistics section 2.3.7, pg. 46). MP generation, as has been previously shown, was cation-dependent.
since it was completely abolished with 10 mM EDTA (MP % = 3.2±0.3; n=13), to the level of that found in resting platelets (< 5%) [18,40,126,127,134]. The approximate 3-4 fold increase in MP generation was determined upon subtraction of background EDTA level from MP generated from WP (MP % = 7.9±0.3) versus MP generated from WP upon the addition of fibrinogen (MP % = 30.6±1.9; p<0.001; by ANOVA; Figure 8b).

Resting washed platelets (MP % = 5.0±0.5) stimulated with human α-thrombin-generated MPs (MP % = 18.4%±0.6; n=3), in the absence of exogenous fibrinogen, in a cation-dependent manner (%MP = 5.7±0.5; n=3; 10 mM EDTA) [18,40,107,126,127]. The generation of MPs was dramatically increased (approximately 4-5 fold) upon the addition of exogenous fibrinogen (100 μg/ml; MPs% = 77.3±0.7; n=3; p<0.001 by ANOVA; Figure 8b). The approximate 4-5 fold increase in MP generation induced by thrombin was determined upon subtracting the background EDTA level (MP% = 12.7±0.7 versus MP% = 71.6±0.8; p<0.001 by ANOVA; Figure 8b).

Further, thrombin-induced MP generation of WP (MP % = 18.4±0.6; n=3) was significantly greater than was SFLLRN-induced WP vesiculation (11.1±0.3; n=13) at the probability level p<0.01, by ANOVA. Furthermore, thrombin-induced WP with added purified fibrinogen vesiculation (MP % = 77.3±0.7; p<0.001; n=3; by ANOVA) was significantly greater than was SFLLRN-induced MP generation in the presence of added fibrinogen (MP % = 33.8±1.9; n=12; Figure 8b).

We hypothesize that the ability of platelets to generate MPs upon activation when washed of all exogenous plasma proteins, could have only been due to the release of internal stores of fibrinogen or of other GPIb/IIIa binding RGD-containing ligands such as fibronectin, vitronectin and vWF. This would explain how WP in the absence of exogenous fibrinogen can generate MPs, albeit to a low level. Nevertheless, the importance of fibrinogen in MP
formation is underscored by the significant increase in MP generation upon the further addition of purified fibrinogen compared to the resting and agonist stimulated WP, irrespective of the agonist used. In addition, human α-thrombin was more efficient in generating MPs than was SFLLRN suggesting a potential role for additional thrombin signalling or an effect of fibrin in washed platelet microparticulization.
Figure 6a Platelets and MPs by flow-cytometry

Resting washed platelets (panels 1 a,b,c), stimulated with SFLLRN (100 μM; panels 2, a,b,c) and incubated with FITC-labelled anti-GPIb monoclonal antibody (panels b, c) are shown in flow cytometric dot plots (forward scatter versus side scatter: panels 1a and 2a) and histograms (fluorescence intensity: panels 1b, c and 2b, c). Platelet specific events, including whole platelets and MPs, were identified as GPIb positive events (Region 1 on panels 1b and 2b). The MP fluorescence gate was set to the immediate right of the background FITC-signal of an irrelevant monoclonal antibody (R1; panels 1b and 2b) and to the left of intact platelets by forward scatter so that maximum number of microparticles, by definition smaller than platelets (< 0.8 μm, based on forward scatter, panels 1a and 2a) were analyzed. Single platelets are to the right of the line (Region 2; panels 1a, 2a). Histograms of single resting (R2) and activated platelets (R2) are shown on panels 1c and 2c, respectively.
Figure 6b MP generation by flow-cytometry

Resting washed platelets with added fibrinogen (100 µg/ml; panels 1), stimulated with SFLLRN (100 µM; panels 2, 3), in the absence (panel 2) and presence (panel 3) of EDTA (10 mM) and saturating concentrations of monoclonal antibodies (FITC-labelled anti-GPIb (panels a) and PE-labelled anti-CD62 (panels b)), are shown in flow cytometric dot plots (forward scatter versus side scatter: panels 1a, 2a and 3a) and histograms (fluorescence intensity: panels 1b, 2b and 3b). Microparticles are smaller than platelets (< 0.8 µm) and to the left of the vertical line while platelets are to the right (panels 1a, 2a and 3a). MPs are reported as a percent of total platelet specific events (panels 1a and 3a<5%; panel 2a>32% MPs). Platelet activation status (M1) or P-selectin surface expression is reported as a percentage of totals (resting WP panel 1b<5%; versus activated WP panels 2b (97%) and 3b (96%)).
Fibrinogen purity was verified by SDS-PAGE. Under non-reduced conditions a single band was observed at 340 kDa correlated to pure fibrinogen (lane 1). Under reduced conditions (lane 2) three bands that correlated to fibrinogen polypeptide chains $\alpha$ (63 kDa), $\beta$ (56 kDa) and $\gamma$ (47 kDa) are seen.
Figure 8a Washed platelet vesiculation induced with SFLLRN

Washed platelets in the presence and absence of pure fibrinogen (FGN; 100μg/ml) and of EDTA (10 mM) were stimulated with 100 μM SFLLRN for 20 min. Samples were then incubated with saturating concentrations of FITC-GPIb monoclonal antibody and MPs were identified by flow cytometry as percent of total platelet specific events (% MP). Highly significant difference in MP generation is observed between washed platelets versus washed platelets upon the addition of pure fibrinogen (n=12-13±SEM; p<0.001*).
Figure 8b Composite bar graph of washed platelet vesiculation induced by SFLLRN or thrombin

WP in the presence and absence of added fibrinogen (FGN; 100 μg/ml) were stimulated with 100 μM SFLLRN or 0.25 U/ml thrombin for 20 min. Samples were then incubated with saturating concentrations of FITC-GPIb monoclonal antibody and MP identified by flow cytometry as percent of total platelet specific events. Presented is composite bar graph of washed platelets generating MP (expressed as % MP subtracted for EDTA background values). %MP induced by SFLLRN (MP%=7.9±0.3; n=13) increased upon the addition of 100 μg/ml fibrinogen (MP%=30.6±1.9; n=11; p<0.001). %MP induced by thrombin (MP%=12.7±0.7; n=3) increased upon the addition of 100 μg/ml fibrinogen (MP%=71.6±0.8; n=3 p<0.001). Significant difference is observed between washed platelet vesiculation induced by SFLLRN versus thrombin (p<0.01*) and highly significant difference between MP generated by SFLLRN versus thrombin after the addition of pure fibrinogen (p<0.001**).
2.4.2 The role of plasma and fibrinogen in MP generation

To further characterize the role of fibrinogen and to evaluate the potential role of other plasma RGD-containing GPIIb/IIIa binding ligands (vitronectin, fibronectin, vWF) in MP formation, we compared the effect of increasing concentrations of purified fibrinogen alone with that of recalcified normal human plasma at known fibrinogen concentrations in supporting MP formation.

Both SFLLRN-activated WP with fibrinogen alone versus plasma with fibrinogen at known concentration generated increasing numbers of MPs in an identical fibrinogen concentration dependent manner (Figures 9a, 9b). SFLLRN-stimulated WP generated 50% of maximum MPs at close to identical fibrinogen concentrations with both fibrinogen sources (p>0.05, no statistical difference by Student's t-test) at OD50=48.3±11.7 μg/ml (n=3) of pure fibrinogen and 58.3±21.8 μg/ml (n=3) of plasma fibrinogen, respectively. Maximal MP generation was approached at 100 μg/ml of added fibrinogen for both exogenous fibrinogen alone and for plasma with native fibrinogen with this agonist (Figure 9a).

Similarly, 50% of maximum MPs was generated with α-thrombin at close to identical fibrinogen concentrations with both fibrinogen sources (p>0.05, no statistical difference by Student's t-test) at OD50=12.5±2.8 μg/ml (n=4) of pure fibrinogen and OD50=11.3±4.5 μg/ml (n=3) of plasma fibrinogen, respectively (Figure 9b). Maximum MP formation occurred at 30 μg/ml for both purified exogenous and diluted (1:30-1:1000) plasma fibrinogen with thrombin as an agonist. Calculation of exogenous fibrinogen concentration dose dependence of MP generation included subtraction of the MPs generated upon WP stimulation with SFLLRN or thrombin (% MP events) relative to maximum of MP generated upon the addition of increasing concentration of fibrinogen and is presented as percent of maximum microparticles generated (% Max MPs).
Thus, MP generation requires fibrinogen and maximal MP formation occurs at 30 μg/ml of fibrinogen with the physiological agonist thrombin and at 100 μg/ml when platelets are stimulated with SFLLRN. These concentrations are well below the physiologic plasma fibrinogen concentration (3 mg/ml) but are consistent with fibrinogen levels required for GPIIb/IIIa saturation with fibrinogen upon platelet activation (100 μg/ml or 0.3 μM) [76,107,184,185]. There was no significant difference between exogenous fibrinogen (purified or plasma fibrinogen) in supporting MP formation. This supports the hypothesis that the contribution of other plasma proteins, including the ligands that bind to GPIIb/IIIa with RGD specificity (vitronectin, fibronectin and vWF) to MP generation is likely minor. Further, these observations may also explain how washed platelets can vesiculate in the absence of added fibrinogen, since endogenous (α-granule) fibrinogen upon secretion and exteriorization can saturate approximately 30% of αIIbβ3 receptors supporting some degree of vesiculation [75,76].

Thrombin was more effective in MP generation than was the PAR1 thrombin receptor activating peptide SFLLRN. Indeed, MP formation by SFLLRN-stimulated platelets required approximately 3-4 fold higher fibrinogen concentration to attain the same level of MP formation (Figures 8b, 9a and 9b). The difference in platelet vesiculation observed by these two agonists, may be explained by the additional enhancing factor(s), that may include cleavage of fibrinogen or the presence of fibrin [12,94,96] or the activity of an additional thrombin-dependent signalling pathway [186].
Figure 9a Effect of exogenous fibrinogen on SFLLRN-induced MP generation

Washed platelets were incubated in the presence of increasing concentrations of fibrinogen (purified or plasma) and stimulated with SFLLRN (100 μM) for 20 min. Samples were then incubated with saturating concentrations of FITC-GPIb monoclonal antibody and MPs were identified by flow cytometry as percent of total platelet specific events. Graph represents exogenous fibrinogen requirement for MP generation. Presented fibrinogen dose dependence of MP generation included subtraction of the base line of MP generated upon SFLLRN WP stimulation in the absence of added fibrinogen. MP generation is reported as percent of maximum generated (% Max MP; n=3±SEM; p>0.05; see text).
Figure 9b Effect of exogenous fibrinogen on thrombin-induced MP generation

Washed platelets were incubated in the presence of increasing concentrations of fibrinogen (purified or plasma) and stimulated with α-thrombin (0.25 U/ml) for 20 min. Samples were then incubated with saturating concentrations of FITC-GPIb monoclonal antibody and MPs were quantified by flow cytometry as percent of total platelet specific events. Graph represents exogenous fibrinogen requirement for MP generation. Presented fibrinogen dose dependence of MP generation included subtraction of the base line MP generated upon WP stimulation with thrombin in the absence of added fibrinogen. MP generation is reported as percent of maximum generated (% Max MP; n=3; p>0.05; see text).
2.4.3 The effect of affinity-purified polyclonal anti-fibrinogen antibodies on washed platelet vesiculation with or without added fibrinogen

We have shown that washed platelets stimulated with thrombin or SFLLRN, without added exogenous fibrinogen, generated MPs to some degree (Figure 8a, 8b). We hypothesized that this ability is due to the presence of endogenous fibrinogen secreted from α-granules. Fully secreted endogenous fibrinogen is known to saturate approximately 30% of GPIIb/IIIa membrane surface receptors upon release [75,76]. The addition of exogenous fibrinogen (purified or plasma) would then allow for greater occupancy of activated surface exposed GPIIb/IIIa, and as we have shown, even greater increase in MP formation (Figure 8a, 8b). However, platelet α-granules are a source of other RGD ligands (vitronectin, fibronectin and vWF) that can bind to GPIIb/IIIa upon thrombin stimulation [76,88,100,184].

To confirm the role of fibrinogen in MP generation and to exclude a potential role for other RGD ligands (both endogenous and exogenous), we evaluated the effect of completely blocking fibrinogen ligand with polyclonal affinity-purified anti-fibrinogen antibodies in washed platelets in the presence and absence of added exogenous fibrinogen or in the physiological milieu of PRP.

As shown in Figure 10a, washed platelets in the absence of exogenous fibrinogen and treated with increasing concentration of polyclonal anti-fibrinogen antibody and SFLLRN, inhibited MP generation in a concentration dependent manner to the level of EDTA treated control samples, while an irrelevant polyclonal antibody had no effect on MP formation. Fifty percent of inhibition (IC50) was observed at 5±0.6 μg/ml of polyclonal anti-fibrinogen antibody (n=3). Polyclonal affinity purified anti-fibrinogen antibody was verified by Cedarlane Labs (Canada), to be monospecific using immunoelectrophoresis and purified fibrinogen. No reaction was seen with normal serum and only one band was observed with
plasma. The inhibitory effect of polyclonal anti-fibrinogen antibody confirms the role of fibrinogen in MP formation and demonstrates that endogenous fibrinogen supports MP formation. Further, these data support the notion that other α-granule-derived RGD ligands (fibronectin, vWF and vitronectin) do not support MP formation. These results also suggest how washed platelets are able to microparticulate to some small degree in the absence of added exogenous fibrinogen.

When 100 μg/ml of purified fibrinogen was added to the washed platelets and incubated with polyclonal anti-fibrinogen antibody and SFLLRN, MP generation was again blocked to the level of the EDTA control (Figure 10b). Inhibition of MP formation was dose-dependent, with 50% inhibition (IC50) at 15.7±1.1 μg/ml (n=4; Figure 10b). An irrelevant polyclonal control antibody against human CD3 had no effect on MP generation (as summarized in Figure 15). Similar inhibition of MP generation was observed with thrombin-stimulated washed platelets and added polyclonal anti-fibrinogen antibody (n=3±SEM; p<0.001 by ANOVA; Figure 10c). Both endogenous and exogenous fibrinogen were blocked by polyclonal anti-fibrinogen antibodies and thrombin-induced MP generation was reduced to the level of EDTA control, while an irrelevant polyclonal CD3 antibody had no effect (p>0.05 by ANOVA; Figure 10c).

In the more physiological setting of PRP, with all of the GPIIb/IIIa binding ligands available, polyclonal anti-fibrinogen antibody completely blocked MP formation in the presence of SFLLRN to the level of the EDTA control, while an irrelevant polyclonal antibody (anti-CD3) did not have any effect on MP generation. This confirms our observations in the purified system (summarized in Figure 16).

These data provide further support to the contention that other RGD ligands (vWF, fibronectin and vitronectin) present either in plasma in the PRP experiments, or secreted from
α-granules in the WP experiments, do not play a significant role in MP generation. While we cannot absolutely exclude any role for other adhesive ligands in MP formation, the blocking of MP generation in conditions close to physiological (PRP) and in WP, by polyclonal anti-fibrinogen antibody, further supports the notion that fibrinogen is the only RGD-containing ligands that plays a central role in the generation of MPs.
Figure 10a Effect of polyclonal anti-fibrinogen antibody on SFLLRN-induced MP formation in washed platelets

Washed platelets were incubated with increasing concentrations of polyclonal affinity-purified anti-fibrinogen antibody and stimulated with SFLLRN (100 µM) for 20 min. Samples were then incubated with saturating concentrations of FITC-GPIb monoclonal antibody and MPs were quantified by flow cytometry. MP inhibition is reported as a percent inhibition calculated relative to the EDTA control. The inhibition of MP formation of WP, by polyclonal anti-fibrinogen antibody, was dose-dependent at IC50=5±0.6 µg/ml (n=3).
Figure 10b Effect of polyclonal anti-fibrinogen antibody on SFLLRN-induced MP formation in washed platelets with added exogenous fibrinogen

Washed platelets, with exogenous fibrinogen (100 μg/ml) were incubated with increasing concentrations of polyclonal anti-fibrinogen antibody and stimulated with SFLLRN (100 μM), for 20 min. Samples were then incubated with saturating concentrations of FITC-GPIb monoclonal antibody and MPs were quantified by flow cytometry. MP inhibition is reported as a percent inhibition calculated relative to EDTA control. The inhibition of MP formation was dose-dependent at IC50=15.7±1.1 μg/ml (n=4).
Figure 10c Inhibition of thrombin-induced MP formation by polyclonal anti-fibrinogen antibody

Washed platelets were incubated in the presence and absence of fibrinogen (FGN; 30 μg/ml), EDTA (10 mM), polyclonal anti-fibrinogen antibody (polycl anti-fgn; 540 μg/ml) and were stimulated by thrombin (0.25 U/ml) for 20 min. Samples were then incubated with saturating concentrations of FITC-GP1b monoclonal antibody and MPs were identified by flow cytometry as percent of total platelet events and presented as percent microparticles (% MP). Presented is a bar graph of the inhibition of MP formation by polyclonal anti-fibrinogen antibody (n=3±SEM, p<0.001 *, see text). Irrelevant polyclonal antibody against CD3 (polycl CD3) had no effect on vesiculation (p>0.05**).
2.4.4 The role of fibrinogen peptides (RGDS and γ chain H12) on washed platelet vesiculation with added fibrinogen and in platelet rich plasma

Fibrinogen binds to the GPIIb/IIIa complex at three sites, including two RGD peptide binding sites (GPIIIa: Aα RGDF 95-97 and Aα RGDS 572-574) that show ligand infidelity for GPIIb/IIIa and H12 (GPIIb: γ400-411), a unique GPIIb/IIIa binding motif. It has been previously shown that the RGDS tetrapeptide completely inhibits MP formation [126] while there are no studies on the effects of γ-chain H12 on MP generation. We sought to further explore the specific role of the fibrinogen binding peptides in MP formation.

Washed platelets (in the presence of 100 µg/ml of purified fibrinogen) were pre-incubated with increasing concentrations of RGDS, γ-chain H12 peptides or mock peptide (GRADSP) then stimulated with SFLLRN. As shown in Figure 11a and Figure 12 both peptides, RGDS and γ-chain H12 completely inhibited MP formation to the level of the EDTA control, in a concentration dependent manner. As summarized in Figure 15, control inactive peptide (GRADSP) did not have any effect on platelet vesiculation. There was no statistical difference between the IC50 of γ-chain H12 (15.33 µM±7.3, n=3; p>0.05 by Student’s t-test) and the IC50 of RGDS (35 µM±15.27; n=3).

Similarly, in a physiological milieu of platelet-rich plasma, both fibrinogen peptides (RGDS and H12) completely blocked platelet vesiculation at near identical concentrations, with IC50=61.66 µM±13.64 (RGDS; n=3; p>0.05 by Student’s t-test) and IC50=73.33 µM±6.6 (H12; n=3), respectively (Figures 11b and 16). The complete inhibition of MP generation with these two anti-fibrinogen peptides provides further evidence that fibrinogen is essential in the process of MP generation.
Figure 11a Effect of RGDS and γ-chain H12 fibrinogen peptides on SFLLRN-induced MP formation in washed platelets

Washed platelets, with added fibrinogen (100 μg/ml), were incubated with increasing concentrations of fibrinogen peptides (RGDS and H12) and were stimulated with SFLLRN (100 μM) for 20 min. Samples were then incubated with saturating concentrations of FITC-GPIb monoclonal antibody and MPs were identified by flow cytometry. The inhibition of MP formation is reported as percent inhibition calculated relative to the EDTA control (n=3; p>0.05, see text).
Figure 11b Effect of RGDS and γ-chain H12 fibrinogen peptides on SFLLRN-induced MP formation in platelet-rich plasma

PRP was incubated with increasing concentrations of fibrinogen peptides RGDS and H12 and stimulated with SFLLRN (100 μM) for 20 min. Samples were then incubated with saturating concentrations of FITC-GPIb monoclonal antibody and MPs were identified by flow cytometry. The inhibition of MP formation is reported as a percent inhibition calculated relative to EDTA control (n=3; p>0.05, see text).
Figure 12 EDTA and H12 inhibition of MP generation assessed by flow cytometry

Washed platelets stimulated with SFLLRN (100 μM) in the presence of 100 μg/ml of fibrinogen (panels 1, 2 and 3), 10 mM EDTA (panels 2) and 0.8 mM γ-chain H12 (panels 3) and incubated with saturating concentrations of monoclonal antibodies (FITC-labelled anti-GPIb (panels a) and PE-labelled anti-CD62P (panels b) are shown in flow cytometric dot plots (panels a) and histograms (panels b). Microparticles are smaller than platelets (< 0.8 μm) and to the left of the line while platelets are to the right (panels a). MPs are reported as a percent of total platelet specific events (panel 1a >32%; panels 2a and 3a<5% MPs). Platelet activation status (M1) or P-selectin surface expression is reported as a percent of total P-selectin positive platelet events (panel 1b=96%; panel 2b=97%; panel 3b=99%).
2.4.5 The role of GPIIb/IIIa receptor in MP generation: anti-GPIIb (P2) and anti-GPIIIa (SZ21) monoclonal antibodies

It has been previously shown that monoclonal antibodies (7E3, A2A9 and PAC-1) against the intact heterodimeric GPIIb/IIIa complex prevent MP generation [89,126]. We further explored the role of the GPIIb/IIIa receptor in MP generation using specific monoclonal antibodies against either GPIIb (clone P2) and/or GPIIIa (clone SZ21) subunits of the GPIIb/IIIa receptor. Neither of these two monoclonal antibodies have been previously evaluated for their effect on MP generation. The epitopes recognized by these two monoclonal antibodies are known to overlap with the fibrinogen binding sites on GPIIb/IIIa molecule and both prevent fibrinogen binding and platelet aggregation [187,188]. The monoclonal antibody against platelet glycoprotein GPIIb (clone P2) binds to GPIIb in the intact GPIIb/IIIa complex and blocks the binding of fibrinogen [187]. The epitope recognized by monoclonal antibody SZ21 (β3 specific) identifies the region of GPIIIa important for both fibrinogen and fibronectin binding [188].

Washed platelets with added purified fibrinogen (100 μg/ml) or PRP were incubated with the agonist SFLLRN in the presence of increasing concentrations of monoclonal antibodies to GPIIb (P2) and GPIIIa (SZ21). MP generation of WP was completely abolished to the level of the EDTA control with these monoclonal antibodies in a dose-dependent manner [IC50=20.5 μg/ml±3.7 (n=4; P2) and IC50=22.5 μg/ml±2.5 (n=4; SZ21); no statistical difference p>0.05 by ANOVA] (Figure 13a). The combination of these two monoclonal antibodies appeared more effective at IC50=9.4 μg/ml±1.2 (n=4; statistically significantly different at p<0.05 by ANOVA) than either used alone (Figure 13a).

Similar effects on MP generation were observed with these same monoclonal antibodies in PRP [IC50=8.5 μg/ml ±1.1 (n=3; P2) and IC50=9.7 μg/ml ±0.8 (n=3; SZ21); no statistical
difference p>0.05, by ANOVA]. The combination of these two monoclonal antibodies was again more effective than either used alone [IC50=4.33 μg/ml ±0.66 (n=3; statistically significantly different at p<0.05 versus P2 or SZ21 alone, by ANOVA; Figure 13b)]. The irrelevant control monoclonal antibody (anti-CD4) had no effect on MP formation in both WP and PRP experiments (Figures 15 and 16). These data confirm the role of the GPIIb/IIIa platelet integrin and its ligand fibrinogen in MP generation.
Figure 13a Effect of GPIIb and GPIIIa monoclonal antibodies on washed platelet MP formation

Washed platelets, in the presence of fibrinogen (100 µg/ml), were incubated with increasing concentrations of anti-GPIIb (anti-CD41; clone P2), anti-GPIIIa (anti-CD61; clone SZ21), either singly or in combination, and were stimulated with SFLLRN (100 µM) for 20 min. Samples were then incubated with saturating concentrations of FITC-GPIb monoclonal antibody and MPs were identified by flow cytometry. The inhibition of MP formation is reported as a percent inhibition calculated relative to the EDTA control. The combination of these two monoclonal antibodies was more effective in the inhibition of MP formation, then either used alone (n=4; p<0.05; see text).
Figure 13b Effect of GPIIb and GPIIIa monoclonal antibodies on washed platelet MP formation in platelet-rich plasma

Platelet-rich plasma (PRP) was incubated with increasing concentrations of anti-GPIIb (CD41; P2), anti-GPIIIa (anti-CD61; SZ21), either singly or in combination, and was stimulated with SFLLRN (100 µM) for 20 min. Samples were then incubated with saturating concentrations of FITC-GPIIb monoclonal antibody and MPs were identified by flow cytometry. The inhibition of MP formation is reported as a percent inhibition calculated relative to the EDTA control. The combination of these two monoclonal antibodies was more effective in the inhibition of MP formation, then either used alone (n=3; p<0.05; see text).
2.4.6 The role of GPIIb/IIIa receptor in MP generation and effect of pharmaceutical agents ReoPro and Integrilin

The ability of RGD peptides to prevent fibrinogen binding to activated platelets has been exploited to create pharmacological anti-GPIIb/IIIa agents with the primary goal of preventing platelet aggregation and platelet thrombus formation \textit{in vivo} [178]. An added role for these anti-platelet agents may be their ability to inhibit MP formation and thus MP procoagulant activity. ReoPro (7E3, abciximab) is the Fab fragment of the chimeric human-murine monoclonal antibody 7E3. 7E3 has been shown previously to inhibit MP formation in gel filtered platelets (GFP) [89,126]. Integrilin (Eptifibatide), a cyclic KGKD containing peptidomimetic, specifically binds the GPIIb/IIIa receptor and prevents fibrinogen and other adhesive ligands from binding, thereby inhibiting platelet aggregation [189]. In the present study we evaluated the role of the GPIIb/IIIa receptor in MP generation using these two anti-GPIIb/IIIa pharmacological agents in the physiological milieu of PRP and in WP with added purified fibrinogen (100 µg/ml).

As shown on Figures 14a and 14b, both anti GPIIb/IIIa drugs were very potent inhibitors of MP generation. The inhibition of MP formation in both PRP and in washed platelets with added fibrinogen was complete and in a dose-dependent manner (p>0.05 by Student’s t-test). Integrilin blocked MP formation with IC50=0.1 µg/ml (or 120 nM) (Figure 14a). Reo Pro blocked MP formation with IC50=1.5 µg/ml (or 31.5 nM) (Figure 14b). When compared on a molar basis, the inhibition of MP formation by ReoPro was 4 fold more potent than that of Integrilin in our experimental conditions of both WP and PRP (p<0.001 by Student’s t-test; Figures 14c and 14d). While ReoPro blocks the binding of adhesive ligands to GPIIb/IIIa and to the vitronectin (avβ3) receptor, Integrilin blocks only GPIIb/IIIa and is platelet specific, implying that its binding site resides on the GPIIb subunit of the heterodimer. These


studies confirmed the role of the GPIIb/IIIa receptor in MP formation and supported our hypothesis that ligand binding is essential for their generation. While ReoPro is neither platelet, GPIIb/IIIa, or ligand specific, Integrilin is specific for the GPIIb/IIIa platelet receptor, underscoring the importance of the GPIIb subunit of heterodimer and of fibrinogen as the required ligand, for the process of MP generation.

Throughout these studies we monitored the extent of platelet activation with a second PE labelled anti-CD62 antibody that detects P-selectin expressed on the surface of the platelet upon activation and the release of α-granule contents. We noted that P-selectin was fully expressed in all experiments with either thrombin or SFLLRN, regardless of the inhibitor used (Figure 12). None of the anti-platelet drugs (ReoPro and Integrilin), EDTA, inhibitory peptides (RGDS and H12) or monoclonal antibodies (P2, SZ21) affected thrombin or SFLLRN-induced platelet activation while they all abolished, in a dose-dependent manner, MP generation (Figures 6b and 12). The implication of this observation is that thrombin (SFLLRN)-induced generation of MPs may proceed independently of the α-granule content release and that platelet activation proceeds undisturbed, regardless of the presence of chelating agents or of GPIIb/IIIa antagonists.

The inhibition of MP formation studies with EDTA, RGDS and γ-chain H12, polyclonal anti-fibrinogen antibody, monoclonal antibodies P2 and SZ21, pharmaceutical agents ReoPro and Integrilin are summarized in Figure 15 (in washed platelets with added purified fibrinogen; n=3-5±SEM; p<0.001 by ANOVA) and Figure 16 (in PRP, n=3-5±SEM; p<0.001 by ANOVA; see statistics, section 2.3.7, pg. 46).
Figure 14a Effect of Integrilin on MP formation in platelet-rich plasma and washed platelets

Washed platelets with added fibrinogen (100 μg/ml) or platelet-rich plasma were incubated with increasing concentrations of Integrilin and stimulated with SFLLRN (100 μM) for 20 min. Samples were then incubated with saturating concentrations of FITC-GPIb monoclonal antibody and MPs were identified by flow cytometry. The inhibition of MP formation is reported as a percent inhibition calculated relative to the EDTA control (n=4; p>0.05).
Washed platelets with added fibrinogen (100 μg/ml) or platelet-rich plasma were incubated with increasing concentrations of ReoPro and stimulated with SFLLRN (100 μM) for 20 min. Samples were then incubated with saturating concentrations of FITC-GPIb monoclonal antibody and MPs were identified by flow cytometry. The inhibition of MP formation is reported as a percent inhibition calculated relative to the EDTA control (n=4; p>0.05).
Washed platelets with added fibrinogen (100 µg/ml) were incubated with increasing concentrations of ReoPro or Integrilin and stimulated with SFLLRN (100 µM) for 20 min. Samples were then incubated with saturating concentrations of FITC-GPIb monoclonal antibody and MPs were identified by flow cytometry. The inhibition of MP formation is reported as a percent inhibition calculated relative to the EDTA control (n=4; p<0.001).
Figure 14d Effect of Integrilin and ReoPro on MP formation in platelet-rich plasma

Platelet-rich plasma was incubated with increasing concentrations of ReoPro or Integrilin and stimulated with SFLLRN (100 μM) for 20 min. Samples were then incubated with saturating concentrations of FITC-GPⅠb monoclonal antibody and MPs were identified by flow cytometry. The inhibition of MP formation is reported as a percent inhibition calculated relative to the EDTA control (n=3; p<0.001).
Figure 15 Composite bar graph of MP inhibition in washed platelets plus fibrinogen

Washed platelets with added fibrinogen (fgn; 100 µg/ml) were incubated in the presence and absence of EDTA (10 mM), ReoPro (100 µg/ml), Integrilin (100 µg/ml), the monoclonal antibodies anti-CD41 (P2; 120 µg/ml), anti-CD61 (SZ21; 120 µg/ml), singly or in combination CD61/41 (120 µg/ml), fibrinogen peptides RGDS (1 mM), γ-chain H12 (0.8 mM), polyclonal affinity-purified anti-fibrinogen antibody (polycl anti-fgn ab; 540 µg/ml) or control peptide GRADSP, monoclonal antibody anti-CD4 (mab anti-CD4) or polyclonal anti-CD3 antibody (polycl anti-CD3), and stimulated with SFLLRN (100 µM) for 20 min at 37 °C. Samples were then incubated with saturating concentrations of FITC-GPIb monoclonal antibody and MPs were identified by flow cytometry and reported as percent of total platelet specific events (%MPs). Presented is a composite bar graph of the inhibition of MP formation in washed platelets with added fibrinogen (n=3-5±SEM; p<0.001 *, while irrelevant controls had no effect on MP generation p>0.05 **, see text).
Figure 16 Composite bar graph of MP inhibition in platelet-rich plasma

Platelet-rich plasma was incubated in the presence and absence of EDTA (10 mM), ReoPro (100 μg/ml), Integrilin (100 μg/ml), monoclonal antibodies anti-CD41 (P2; 120 μg/ml), anti-CD61 (SZ21; 120 μg/ml), singly or in combination CD61/41 (120 μg/ml), fibrinogen peptides RGDS (1 mM), γ-chain H12 (0.8 mM), polyclonal anti-fibrinogen antibody (poly cl anti-fgn AB; 540 μg/ml), control inactive peptide GRADSP, monoclonal antibody anti-CD4 (mab anti-CD4) or polyclonal anti-CD3 antibody (poly cl anti-CD3), and stimulated with SFLLRN (100 μM) for 20 min at 37 °C. Samples were then incubated with saturating concentrations of FITC-GPIb monoclonal antibody. MPs were identified by flow cytometry and reported as percent of total platelet specific events (%MPs). Presented is a composite bar graph of the inhibition of MP formation in PRP (n=3-5±SEM; p<0.001 *, while irrelevant controls had no effect on MP generation p>0.05 **, see text).
2.5 Discussion

MP formation is a normal physiological consequence of platelet activation and is observed in a wide range of clinical thrombocytopenic and arterial and venous prothrombotic conditions, yet the mechanism(s) of MP generation remain unclear [6,21,108,147,148,190]. It has been previously shown that the GPIIb/IIIa receptor plays a key role in platelet microparticle generation, as Glanzmann's thrombasthenia type I platelets were markedly impaired in their ability to generate MPs, and the RGDS peptide and monoclonal antibodies against the GPIIb/IIIa complex inhibited MP formation [89,126]. These observations suggested that one or all of the GPIIb/IIIa RGD-containing ligands (fibrinogen, vitronectin, fibronectin and vWF), could play a role in platelet vesiculation [126].

We have further defined the role of fibrinogen and of the β₃ platelet integrin GPIIb/IIIa in thrombin-induced MP formation. We compared, using flow cytometry, the ability of PRP (as a source of exogenous fibrinogen and other plasma proteins including RGD specific ligands vitronectin, fibronectin and vWF) to the ability of a platelet suspension that was completely washed free of all plasma proteins, to generate MPs. The role of exogenous fibrinogen in MP generation by washed platelets was further probed by the addition of increasing concentrations of purified fibrinogen or of recalcified human plasma at a known fibrinogen concentration. Fibrinogen peptides, RGDS and γ-chain H12, polyclonal anti-fibrinogen antibody, monoclonal antibodies against GPIIIa (P2) or GPIIIa (SZ21) and pharmaceutical agents ReoPro and Integrilin further probed the role of fibrinogen in MP generation.

We have demonstrated that only fibrinogen, with its RGD and γ-chain dodecapeptide binding sites, is essential for MP generation and that other RGD ligands present in plasma or
α-granules (vitronectin, fibronectin and vWf) are not. We have further confirmed the central role of the GPIIb/IIIa receptor in MP formation.

2.5.1 The role of fibrinogen in MP generation

2.5.1.1 The role of purified fibrinogen in MP generation

Fibrinogen is the dominant physiological platelet integrin GPIIb/IIIa ligand, with a central role in normal hemostasis in supporting platelet aggregation and fibrin formation [81]. Fibrinogen is also one of the family of RGD-containing, GPIIb/IIIa binding ligands that include vitronectin, fibronectin and von Willebrand factor [88,101]. The RGD-GPIIb/IIIa receptor-peptide paradigm has been well established with the discovery that fibronectin's binding site peptide RGD inhibits both fibrinogen binding to the GPIIb/IIIa integrin and platelet aggregation [100,191]. We now recognize that there are a number of ligands (including vitronectin, fibronectin, vWf, collagen, prothrombin, thrombospondin and disintegrins such as barbourin) and integrin receptors (including αvβ1, αvβ3, αvβ5, α3β1, α5β1, α8β1 and αvβ6) that share a common RGD recognition specificity [43].

Washed platelets stimulated with thrombin or SFLLRN are capable of generating MPs. However, a significant increase (approximately 3-4 fold) in MP formation occurred with the addition of exogenous fibrinogen, either purified (30-100 μg/ml) or plasma-derived (30-100 μg/ml) to washed platelets (Figures 8, 9a and 9b). This indicated that there is a specific ligand requirement in the process of platelet microparticulization and that this ligand is most likely fibrinogen. The concentration of the internal α-granule pool of fibrinogen has been reported to range between 7-14 μg/10⁸ platelets. This is sufficient, when fully released upon activation, to occupy approximately 30% of the cell surface GPIIb/IIIa molecules [43,75,76,184]. In our experiments with 3-3.5x10⁸/ml platelets, the maximal total release of
internal fibrinogen pool would be 21-49 μg/ml. This amount of fibrinogen was evidently sufficient to elicit the modest generation of MPs upon SFLLRN stimulation in the absence of added fibrinogen (Figure 8a). Further vesiculation occurred when exogenous purified fibrinogen was added. Maximal washed platelet vesiculation occurred at 30 μg/ml of exogenous fibrinogen when platelets were stimulated with human α-thrombin and at 100 μg/ml when they were stimulated with SFLLRN. 100 μg/ml has been reported to be the saturating concentration of fibrinogen to stimulated platelets [75,76,107,184]. This suggests that maximal MP formation requires close to full GPIIb/IIIa occupation but that MP formation can still occur, albeit to a lower extent, with a lower GPIIb/IIIa occupancy.

Thrombin had an enhanced effect on platelet vesiculation when compared to its receptor activating peptide SFLLRN. Thrombin-induced MP formation required approximately 4-5 fold lower fibrinogen concentration as did SFLLRN, suggesting the existence of additional thrombin-dependent signalling on platelet activation or some other thrombin-dependent function. Thrombin signalling is initiated through G-protein coupled "protease-activated receptors" termed PAR1 and PAR4 [186]. Thrombin receptor activating peptide SFLLRN activates PAR1 without proteolysis while having no activity on PAR4 [186]. Additionally, other receptors may act as thrombin activation site such as the GPIb/IX/V receptor [192,193]. Alternatively or in addition to this, thrombin cleavage of fibrinogen or the presence of fibrin binding to GPIIa/IIIa [12,94,96] could have potential influence on platelet function in generating MPs [81].
2.5.1.2 The role of purified fibrinogen versus plasma at a known fibrinogen concentrations

MP generation was fibrinogen dose-dependent, consistent with the concept that the degree of MP formation is directly related to the extent of GPIIb/IIIa occupancy. There was no difference between dose-dependency curves generated with purified fibrinogen or with added plasma at a known fibrinogen concentration, irrespective of the agonist used. The plasma experiments support the hypothesis that fibrinogen is the only RGD plasma protein that supports MP formation since vitronectin, fibronectin and vWf are also available in plasma to potentially occupy GPIIb/IIIa [87,88,178], but do not support MP formation.

Fibrinogen is present in plasma at an abundant concentration of ~3 mg/ml (7-9 μM), ensuring full GPIIb/IIIa occupancy. MP generation in vivo can therefore be supported at any given time, irrespective of the agonist, as long as the GPIIb/IIIa receptor is intact. GPIIb/IIIa receptor occupancy is dependent upon stimulatory signals and is dictated by the demands for hemostasis [48]. Therefore, platelet activation in the circulation induced by weak agonist (ADP, TxA2, epinephrine) would be expected to result in minimal MP generation, while thrombin platelet activation would result in an increased number of MPs, consistent with the increased level of GPIIb/IIIa activation and the higher number of exposed activated GPIIb/IIIa receptors [39,40,127,138].

Further, patients with congenital afibrinogenemia bleed intensely after even minor trauma. Despite normal platelet counts and normal levels of other plasma proteins their thrombin clotting time is infinite [9,194,195]. Platelets from these patients lack the internal pool of fibrinogen, while α-granule von Willebrand factor and fibronectin stores are intact [194,195]. To the best of our knowledge, there have been no studies that addressed the ability of the platelets from afibrinogenemic patients to generate MPs. However, the aggregation of
platelets from these patients has been variably reported as normal or impaired [196,197].
The bleeding tendency is usually ameliorated if a plasma level of 0.5 mg/ml of fibrinogen is
attained to the patients [9]. Afibrinogenemia, as the natural experiment, could provide
further insights into the role of fibrinogen and of other plasma proteins and RGD ligands in
the process of MP formation.

A question remains as to whether there is a difference between the plasma-derived and the
internal (α-granular) fibrinogen pool in MP formation. Earlier research that suggested that
endogenous fibrinogen is different from plasma-derived fibrinogen has been refuted by
several lines of evidence. Plasma fibrinogen is taken up into α-granules, by either
megakaryocytes or platelets, through GPIIb/IIIa cycling, indicating that there is no molecular
difference between these two fibrinogen sources, since they both ultimately derive from a
common plasma source [43,48,75,77,78,198]. Fibrinogen internalisation and cycling of the
GPIIb/IIIa receptor appear to be constitutional regulatory mechanisms that modulate platelet
function by adjusting availability of GPIIb/IIIa receptor and other proteins from α-granules
according to the needs for hemostasis [48].

2.5.2 The role of affinity-purified polyclonal anti-fibrinogen antibodies: fibrinogen
versus other RGD-containing binding ligands

We confirmed the central role of fibrinogen in MP generation by demonstrating full
inhibition of washed platelet vesiculation by polyclonal anti-fibrinogen antibodies. Other
ligands that bind GPIIb/IIIa with RGD specificity and that are released together with
fibrinogen from α-granules upon washed platelet stimulation with thrombin or SFLLRN
(vitronectin, fibronectin or vWF) [76], did not support MP generation after fibrinogen binding
was blocked with polyclonal anti-fibrinogen antibodies. Others have shown that endogenous
fibronectin, vWF and vitronectin, (present in α-granules at 0.3 μg/10^8, 0.2 μg/10^8 and 0.8 μg/10^8 platelets, respectively), are surface expressed, and are bound to thrombin-activated GPIIb/IIIa receptors unoccupied by fibrinogen or in the absence of fibrinogen [9,76,88,199]. We completely blocked MP generation of washed platelets with polyclonal anti-fibrinogen antibodies while an irrelevant polyclonal antibody had no effect. Thus, the generation of MPs in the absence of added exogenous fibrinogen is supported by fibrinogen secreted from platelet α-granules, and not by other RGD ligands, indicating an absolute fibrinogen requirement for MP generation.

It is generally accepted that the role of the internal fibrinogen pool in normal hemostasis is the local concentrated release of fibrinogen at the site of vascular injury, to provide for platelet aggregation and fibrin formation [9,75,76]. It was recently reported that the internal pool of GPIIb/IIIa receptors, situated in α-granules and the inner lining of open canalicular system (OCS) undergoes conformational changes upon platelet stimulation by thrombin (but not ADP) and that internal pool of fibrinogen could emerge to the platelet membrane surface, already bound to activated GPIIb/IIIa [53,200]. If this is the case, our data suggest that such fibrinogen binding is reversible since MP generation of washed platelets was fully blocked by polyclonal anti-fibrinogen antibodies. The observation that, while other RGD-containing internal ligands would have been available to bind to GPIIb/IIIa receptor in our experiments, but MPs were not generated, suggests that such ligands may have an inhibitory (regulatory) role in MP generation. RGD ligands bind to activated GPIIb/IIIa in competition with fibrinogen, via their common RGD recognition sequence but do not interact with the γ-chain binding site, unique to fibrinogen. It is possible, therefore, that these ligands may not only block fibrinogen binding, but may also induce different conformational changes in GPIIb/IIIa than the changes induced by fibrinogen, thereby sending “outside-in” signalling that may
regulate MP generation and modulate procoagulant platelet activity. To this date, the role of
the α-granular internal pool of RGD ligands (that includes fibrinogen, fibronectin, vitronectin
and von Willebrand factor) in hemostasis has not been fully explained [76,88,98].

The significance of, and the requirement for fibrinogen in MP generation was further
demonstrated by the complete, dose-dependent inhibition of MP formation by thrombin or
SFLLRN-stimulated platelets in the presence of exogenous purified fibrinogen and in
physiological milieu of PRP by polyclonal anti-fibrinogen antibody (Figure 10c, 15 and 16). RGD ligands released from the internal pool, or present in plasma did not support MP
generation after exogenous (purified or plasma) fibrinogen was eliminated by polyclonal
antibody.

Plasma constitutes a significant exogenous source of fibrinogen (7-9 μM; with Kd 0.17-
0.3 μM), fibronectin (0.7 μM with Kd 0.3 μM), vitronectin (2.3 μM) and smaller
concentrations of von Willebrand factor (monomeric, 11 nM) all able to bind to thrombin-
induced platelet GPIIb/IIIa receptors unoccupied by fibrinogen and in the absence of
fibrinogen (afibrinogenemia) [9,12,76,88,98,184]. Plasma fibrinogen concentration is in a
20-30 fold excess of the Kd for its GPIIb/IIIa receptor occupancy, providing for full receptor
saturation upon thrombin stimulation. Thus, fibrinogen is the primary GPIIb/IIIa binding
ligand [76,88,184]. In both WP and PRP experiments, inhibition of fibrinogen binding
resulted in complete inhibition of MP generation despite the presence of the RGD binding
ligands available from α-granules. Thus, these observations, together with our studies using
purified fibrinogen or plasma, identified fibrinogen as the only RGD-containing GPIIb/IIIa-
binding ligand that was required for platelet microparticle generation. In addition, our data
are consistent with a potential inhibitory role of other RGD-containing ligands in MP
formation. Further experiments are needed to elucidate the functional role(s) in MP
generation of the binding of other RGD ligand (fibronectin, vitronectin and vWF) to GPIIb/IIIa.

2.5.3 The role of fibrinogen peptides RGD and H12 in MP generation

Fibrinogen binds to GPIIb/IIIa via two RGD sequences located on each pair of α-chains (RGDF at 95-97 and RGDS at 572-574) and an additional binding site on the γ-chains (H12 at 400-411) [82-85]. Previous research has shown that RGDS peptide inhibits MP formation [89,126]. There are no data on the role of γ-chain H12 peptide in MP formation. Previous studies that employed recombinant mutant fibrinogens (rFbg) defective in RGD and γ-chain H12 binding sites have indicated that γ-chain H12, as opposed to RGD, has an essential role in platelet aggregation and adhesion [90,91]. Mutant rFbg with an RGD/RGE substitution at either α-chain 97 or α-chain 574 (rFbg-D97E; rFbg-D574E) did not demonstrate an impaired ability to aggregate ADP stimulated platelets [90]. In contrast, recombinant fibrinogen defective in the γ site (rFbg-γ') was markedly deficient in its ability to mediate platelet aggregation and adhesion [91]. Further, binding studies using peptide and a specific monoclonal antibody against fibrinogen AGDV γ-chain sequence (clone 4A5), located the role of fibrinogen γ-chain H12 in binding to GPIIb/IIIa complex, platelet aggregation and adhesion to the carboxy terminal AGDV sequence of fibrinogen γ-chain (408-411) [93-97]. These studies demonstrated directly that the RGD sequence is not necessary, but that γ-chain H12 is critical for platelet aggregation and adhesion.

The carboxy terminus γ-chain H12 is unique to the fibrinogen molecule and binds to platelet specific GPIIb subunit, while other vascular ligands can bind to the GPIIIa subunit of β3 platelet integrin with RGD sequence [43]. Ours is the first study to evaluate the potential role of the fibrinogen γ-chain H12 in MP formation and to demonstrate that MP formation is
γ-chain H12 dependent. Furthermore, both RGDS and γ-chain H12 peptides inhibited MP formation of washed platelets (in the presence and absence of added fibrinogen) and in PRP in a dose-dependent manner, at similar molar concentrations (Figure 11a and 11b). The inhibition of MP formation in the presence of purified fibrinogen correlated approximately with reported IC50 of 125I-fibrinogen binding to ADP stimulated human platelets by RGDS (15.6±2.7 µM) [86] and γ-chain H12 peptides (28 µM) [84]. Since the γ-chain H12 binding site of fibrinogen has been shown to be essential for platelet aggregation [90,91,93,95,97], the additional role of this binding site in MP formation suggests a relationship and potential dependence, through “outside-in” GPIIb/IIIa signalling and GPIIb/IIIa clustering, of the platelet aggregation process and MP shedding [19,29,30,43,49,201-203].

Fibrinogen RGD and γ-chain H12 peptides bind to resting GPIIb/IIIa and block the binding of RGD ligands (fibrinogen, fibronectin, vitronectin and vWF) but induce GPIIb/IIIa activation, acting at the same time as an agonist and an antagonist [84-88,177,199,202]. We and others have not seen evidence that these peptides, can induce MP generation [89,156]. Further, the binding to the GPIIb/IIIa receptor of the fibrinogen peptides (RGD and H12) has been reported to be mutually exclusive due to the activation induced conformational change of GPIIb/IIIa that excludes simultaneous residing of both peptides on GPIIb/IIIa [86,177]. It has recently been reported that high affinity cyclic analogues for the RGD and H12 peptides induced different conformational changes in GPIIb/IIIa receptor upon binding and different changes in platelet membrane fluidity implying distinct “outside-in” signalling by these peptides [201]. A possible explanation of why fibrinogen supports MP formation yet the other RGD binding macromolecules do not, may reside in the γ-chain binding site, since different physico-chemical effects have been demonstrated by others, when RGD or H12 cyclic peptides occupy the GPIIb/IIIa receptor [201]. In addition, multiple contacts of
fibrinogen with GPIIb/IIIa receptor may play the role since fibrinogen peptides alone (RGD or H12) do not support MP generation. Thus, the inhibition of MP formation, demonstrated by polyclonal anti-fibrinogen antibody experiments and with the inhibition of MP formation by γ-chain H12 studies, support the postulate that fibrinogen is the only RGD ligand that supports MP generation in platelets. Further, we hypothesize that the cryptic AGD sequence of γ-chain H12 is the critical motif that distinguishes fibrinogen from other RGD specific binding ligands in supporting MP generation [95,97]. Further study is needed to elucidate the relative role(s) of fibrinogen's binding sites RGD and γ-chain H12 in the process of MP generation.

2.5.4 The role of the GPIIb/IIIa receptor in MP generation

2.5.4.1 The effect of anti-GPIIb and anti-GPIIIa monoclonal antibodies

Previously, we and other researchers demonstrated that monoclonal antibodies 7E3, A2A9, 10E5 (αIIbβ3 complex specific) blocked MP formation in GFP [89,126,204]. Further, the congenital bleeding disorder, Glanzmann's thrombasthenia type I, which is characterized by the inability of platelets to aggregate due to a deficiency of functional GPIIb/IIIa receptor, is also characterized by the impaired MP generation [44]. Studies with Glanzmann's thrombasthenia platelets provided the first direct evidence that the β3 integrin platelet GPIIb/IIIa receptor plays a central role in the process of microparticle generation [89,126]. The platelets of these patients also lack intra-granular fibrinogen, a deficiency that confirms a role for the GPIIb/IIIa receptor in the trafficking of plasma proteins in and out of platelet α-granules [198]. However, it appears that thrombasthenic platelets are able to vesiculate when activated through the complement system, suggesting a possible different mechanism of MP generation via complement [173]. Consistent with this notion, Nomura et al., identifying the
participation of the GPIIb/IIIa receptor in the MP formation induced by thrombin and collagen, also suggested that there exist two distinct mechanisms of MP generation, only one of which is GPIIb/IIIa dependent [205].

The central roles of the GPIIb/IIIa receptor and of fibrinogen in MP formation were further confirmed by our studies using monoclonal antibodies against GPIIb and GPIIIa subunits [187,188]. We demonstrated that MP generation of washed platelets (in the presence and absence of purified fibrinogen), and of PRP upon SFLRN stimulation is blocked by specific monoclonal antibodies against either or both the GPIIb (clone P2) and GPIIIa (clone SZ21) subunit. While these two antibodies also inhibit fibronectin binding, we have already demonstrated that fibronectin and other RGD ligands do not support MP formation. Further, we demonstrated that there was no difference between these two specific monoclonal antibodies in the inhibition of MP formation when used separately. In combination, however these monoclonal antibodies where more potent having an additive inhibitory effect on MP generation. This observation suggests that these two antibodies may prevent each other from binding simultaneously to the GPIIb/IIIa receptor. This is similar to fibrinogen peptides RGD and γ-H12, whose binding to GPIIb/IIIa has previously been shown to be mutually exclusive due to binding induced GPIIb/IIIa conformational changes and with an additive inhibitory effect on fibrinogen binding [82,86,177]. Binding of the P2 monoclonal antibody has previously been shown by PAC-1 monoclonal antibody studies not to induce activation changes of the GPIIb/IIIa receptor, while the effect of SZ21 monoclonal antibody on conformational changes of the GPIIb/IIIa receptor, to the best of our knowledge, is not known [206]. We speculate that binding of different GPIIb/IIIa inhibitors may trigger distinct changes in the GPIIb/IIIa receptor that may result in different functional consequences. Each of these two monoclonal antibodies recognizes a different subunit
(SZ21 binds to β3, while P2 binds to αIIb subunits in an intact complex) and have not been used previously in studies of MP generation. We have confirmed, with these two monoclonal antibodies, the key role of the GPIIb/IIIa receptor in MP generation.

2.5.4.2 The effect of the pharmacological agents ReoPro and Integrilin

In recent years, treatment and clinical outcomes in acute coronary syndromes (ACS), myocardial infarction (MI) and after coronary revascularization (PTCA) have improved significantly with the clinical use of anti-platelet GPIIb/IIIa blocking agents [207]. These drugs target the GPIIb/IIIa receptor that result in the blocking of fibrinogen binding, which compromises platelet aggregation and adhesion at sites of vascular injury. Most of these drugs have been modelled according to the RGD prototype sequence and the RGD ligand-integrin paradigm [178]. It is generally accepted that the primary anti-thrombotic effect of these agents is through the prevention of and interference with platelet aggregation and adhesion at arterial vascular thrombotic sites.

ReoPro (7E3, abciximab) is the Fab fragment of the chimeric human-murine anti GPIIb/IIIa platelet monoclonal antibody 7E3 that binds only to the intact GPIIb/IIIa complex [89,99]. The mechanism of prevention of fibrinogen binding and platelet aggregation with abciximab, is believed to be due to the conformational induced effects that block the access of large molecules (fibrinogen, von Willebrand factor and other adhesive ligands) to the integrin receptor, rather than due to the direct binding to and interaction with the RGD binding site on GPIIb/IIIa. ReoPro also binds to the vitronectin receptor shared by platelets, endothelial cells and smooth muscle cells, as well as to leukocyte αMβ2 (Mac-1, CD11b/CD18) [65]. It is not clear from current clinical data if this lack of abciximab
specificity is beneficial or harmful. It has been observed in clinical practice that the use of ReoPro is accompanied with rare but not completely explained thrombocytopenia [179].

Integrilin, a cyclic peptidomimetic that contains the KGD sequence was created based on the chemistry of barbourin, a disintegrin [189,208]. Integrilin binds to GPIIb part of β3 platelet integrin thereby it is platelet specific. Integrilin prevents binding of adhesive ligands (fibrinogen, von Willebrand factor and other adhesive ligands) and platelet aggregation [189].

We have shown that both fibrinogen peptides (RGD and γ-H12), anti-GPIIb/IIIa drugs (ReoPro and Integrilin) and monoclonal antibodies against GPIIb (P2) and GPIIIa (SZ21) are able to completely inhibit MP generation, consistent with our observation of the central role for both the GPIIb/IIIa receptor and fibrinogen in MP formation. We have demonstrated that platelet activation was undisturbed by these agents, as judged by complete platelet degranulation (P-selectin expression) and αIIbβ3 upregulation, indicating that α-granules release is not essential for MP formation. However, the question remains as to the effect of these inhibitors on platelet procoagulant activity that is related to the exposure of negatively-charged aminophospholipids (PS) at the outer membrane leaflet and is accompanied by the shedding of procoagulant MPs [21,40,166,209]. We postulate that by inhibiting MP formation following platelet activation, these drugs will lead to the elimination of an important catalytic surface for thrombin generation. Reverter et al. demonstrated that the reduction in thrombin generation, that results from the binding of antibody 7E3 to the GPIIb/IIIa receptor is accompanied by the inhibition of MP formation [89]. This was in agreement with our previous work and our current postulate that the generation of MPs as a surface for the assembly of coagulation factors requires the GPIIb/IIIa receptor [126,203]. A recent study demonstrated that exposure of negatively-charged phospholipids on the surface
of activated platelets, was reduced in the presence of the monoclonal antibody against the GPIIb/IIIa [210]. In addition, washed platelets activated with thrombin and pretreated with RGDS peptide had reduced exposure of negatively-charged phospholipids on their surface and reduced "bleb" formation upon adhesion to fibrinogen and fibrin-coated surfaces [211]. Reduction in platelet surface factor V/Va binding and reduced exposure of negatively charged phospholipids in whole blood, were directly linked to the GPIIb/IIIa receptor and its binding ligand, since this reduction was observed with platelets treated with ReoPro, Integrilin and Aggrastat [174]. Other researchers also found a relationship between the GPIIb/IIIa receptor, GPIIb/IIIa blockers and thrombin generation [89,204,212]. We suggest that ReoPro and Integrilin, used primarily as anti-platelet agents mediate an additional anticoagulant effect in vivo by the inhibition of MP formation, and thereby of MP-related procoagulant activity.

2.5.5 Conclusions

In summary, we have proven our hypothesis that thrombin or SFLLRN-induced MP generation has an absolute requirement for fibrinogen and for the GPIIb/IIIa receptor. Further, we have demonstrated that both fibrinogen peptides, GPIIIa subunit binding RGDS and GPIIb subunit binding γ-chain H12, play an essential role in MP generation. In contrast, other RGD-containing ligands (vitronectin, fibronectin and vWF) do not support platelet microparticulization.

We have also shown that endogenous fibrinogen alone can support MP formation in the absence of an exogenous source. Maximum MP generation, however, is observed following thrombin or SFLLRN platelet stimulation, in the presence of 30 μg/ml and 100 μg/ml, respectively, of added fibrinogen. In contrast, other RGD-containing ligands, both
endogenous and exogenous do not support MP generation. Their potential regulatory role(s) in MP generation warrants further study.

We also characterized the role of the fibrinogen specific, GPIIb binding γ-chain H12, in MP generation. We suggest that the GPIIb part of the β3 integrin GPIIb/IIIa (which contains the binding site for fibrinogen γ-chain H12 and AGD sequence) is critical for MP generation, since other RGD ligands that can occupy the GPIIb/IIIa receptor do not support MP formation. We suggest that other RGD ligands (fibronectin, vitronectin and vWF) may play a regulatory role in platelet procoagulant activity via the inhibition of MP formation. Further study is needed to elucidate the relative functional role(s) in MP generation of fibrinogen's binding sites RGD and γ-chain H12.

Thrombin is a more potent MP inducer than is peptide SFLLRN. This observation warrants further study.

The GPIIb/IIIa blocking anti-platelet drugs, ReoPro and Integrilin, inhibit MP generation. We suggest that the inhibition of MP generation thereby abrogates the function of an important catalytic surface for coagulation activation. Thus, the GPIIb/IIIa blocking anti-platelet drugs, ReoPro and Integrilin also function as anti-coagulant agents. We therefore suggest that the use of these GPIIb/IIIa blocking agents could be expanded from current use in interventional cardiology to include other clinical conditions characterized by platelet activation, blood vessel wall injury or platelet procoagulant activity.
"Biological properties of platelet-derived-microparticles: Microparticle adhesion to immobilized fibrin, fibrinogen and collagen type I"

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Part of this work: "Surface adhesion properties of platelet-derived microparticles" has been presented as posters at the:

Annual Meeting of the American Society of Hematology, New Orleans, Dec 1999

Hematology Days, Toronto Jan 2000


Part of this work: "Biology of platelet-derived microparticles: adhesion to fibrinogen, fibrin and collagen" was orally presented at the International Congress of Thrombosis and Haemostasis, Paris, July 2001.

An abridged version of this chapter will be submitted in manuscript form to the journal of the American Society of Hematology "Blood".
Chapter 3 Adhesive properties of platelet MPs

3.1 Abstract

Platelet-derived microparticles (MP) are activation dependent, procoagulant membrane microvesicles observed in thrombocytopenic and thrombotic states. The adhesive properties of MPs, unlike those of platelets, are poorly understood. We have characterized and compared the adhesion of washed human platelets (WP) and purified MPs to surface-bound proteins (albumin, fibrinogen, fibrin and collagen type I), in a unique, static, microsphere adhesion assay. Biotinylated, SFLLRN-generated MPs, isolated from platelets (96.6±1.0% pure) by differential centrifugation and filtration (0.8 µm filter), or resting biotinylated WPs, were incubated with protein-coated microspheres (PCM) (10 µm), and surface-bound MPs or WPs were quantitated by flow cytometry (Fl. intensity) and visualized by scanning (± immunogold) electron microscopy (SEM). MP and WP adhesion to PCM were compared relative to their adhesion to control albumin-coated microspheres (CM; n=4-7±SEM). MP adhesion was greatest to fibrin (9.2±2.5X), then fibrinogen (4.3±0.7X), collagen (4.2±0.8X) PCMs and plain microspheres (1.3±0.2X) versus albumin CM. WP adhesion to PCMs was contrasted in parallel studies: greatest on fibrinogen, then fibrin, collagen and uncoated beads (32.3±7.4X, 24.3±4.7X, 13.9±2.1X, 1.8±0.2X, respectively) versus albumin. SEM of MPs or WPs on PCMs confirmed the results from the flow cytometric studies. Adherent MPs were flattened, spherical and singular (0.1 µm on average) or surface-bound clusters of aggregates (0.2-0.8 µm). Immunogold SEM of surface-bound MPs confirmed their platelet origin. MP and WP adhesion to all surfaces was abolished with 10 mM EDTA. MP and WP adhesion to PCM was ligand and receptor specific. Adhesion of MPs to fibrinogen and fibrin PCMs was
fully inhibited by GPIIb/IIIa agents (Integrilin and ReoPro) and by polyclonal anti-fibrinogen antibody. Adhesion to collagen surfaces was completely inhibited by polyclonal anti-collagen antibody and by a collagen receptor (GPIa/IIa) monoclonal antibody. SEM confirmed these receptor-ligand observations. We conclude that MPs adhere specifically to fibrinogen, fibrin and collagen via functional receptor-ligand complexes by mechanisms similar to those of platelets. Furthermore, we demonstrate that MPs aggregate and expand the role of ReoPro and Integrilin to include inhibition of MP adhesion to immobilized fibrin and fibrinogen. Studies are needed to define adhesion of MPs under flow conditions and to other platelet binding surface ligands including vWF, fibronectin and vitronectin.
3.2 Introduction

Platelet adhesion to vascular ligands (in addition to aggregation, activation and shedding of procoagulant microparticles), is a physiological response of platelets to the injured blood vessel wall, with a major role in preserving the integrity of the blood vessel [2,18]. Adhesion involves the binding of distinct platelet membrane receptors to immobilized or soluble ligands: GPIIb/IIIa to fibrinogen, fibrin, vitronectin and fibronectin, GPIa/IIa to collagens, αvβ3 to vitronectin, GPIc/GPIIa to fibronectin under low shear forces [10,11,23], and GPIIb/IIIa to fibrinogen and vWF and GPIb to vWF under high shear forces [12,27]. However, in diseases characterized by endothelial and subendothelial injury and the deposition of fibrinogen (fibrin), platelet activation, adhesion and shedding of microparticles occur, resulting in growing thrombi with a potential for vascular occlusion, embolization or the dissemination of procoagulant activity [3]. Platelet activation is one of the underlying mechanisms of vascular-atherosclerosis and acute arterial thrombosis, that result in myocardial infarction, transient ischemic attacks or cerebral infarction, the major causes of death in the western world [2].

Platelet-derived microparticles are platelet activation events first described in 1967 as "platelet dust" [106]. Their minute size (less than 0.8 μm) initially restricted their research. However with the introduction of modern flow cytometry techniques, the assessment of MP biology is now possible [107]. The membrane content of MPs is similar to that of platelets, and GPIIb/IIIa, GPIb and P-selectin are regularly detected on their surface by flow cytometry. MP membranes express negatively-charged aminophospholipids rich in binding sites for coagulation factors Va and VIIIa [21,40,144].

There is a growing body of clinical evidence supporting the significance and procoagulant role of MPs since elevation of MPs in the circulation has been correlated with a wide range
of clinical thrombocytopenic and thrombotic conditions associated with platelet activation and thrombosis (ITP, TTP, HIT, MI, PTCA, DVT, DIC, sepsis) [6-8,114-122].

While the study of platelet physiology, biochemistry and role in hemostasis and thrombosis is well characterized, the role in the circulation and the biological properties of platelet-derived MPs, remain unresolved. Therefore, it is essential to define the biological similarities or differences of MPs and platelets to better understand the role of MPs in the circulation. With respect to their adhesive properties and their abilities to coaggregate to other MPs and to platelets or other cells, very little is known. Reports suggest that MPs are able to bind to fibrin and fibrinogen [136,168], endothelial cells [154], subendothelial matrix [169] or to interact with other cells [171,172]. Other reports have noted qualitative differences between the platelet GPIIb/IIIa receptor and of the MP membrane GPIIb/IIIa receptor, suggestive of the inability of MPs to adhere or aggregate via the GPIIb/IIIa receptor (or to react with other cells through GPIIb/IIIa) [170]. An issue exists in these studies as to the definition of MPs and whether there is contamination with remnant small platelets in the material assessed as MPs. This issue is complicated by the inability of flow cytometry techniques to absolutely separate platelets from generated MPs and to identify MPs smaller than 0.1-0.2 μm, otherwise regularly seen by SEM [131].

In this study we sought to elucidate whether MPs and platelets have similar adhesive properties to the vascular ligands that play a role in platelet adhesion (fibrinogen, fibrin and collagen type I). We characterized and compared the adhesion of washed platelets (WP) and purified MPs to surface-coated proteins (albumin, fibrinogen, fibrin and collagen type I) using a unique, static, microsphere-based flow cytometric adhesion assay. Findings were corroborated by scanning electron microscopy imaging (SEM) and by immunogold SEM. Our investigations suggested that the adhesion of MPs to fibrinogen, fibrin and collagen type
I is similar to that of platelets. In addition, we demonstrate that MP adhesion is metal-ion dependent, receptor and ligand specific, similar to platelet adhesion.

3.3 Methods and materials

3.3.1 Materials

Bovine serum albumin (BSA), human α-thrombin, EDTA, prostaglandin E₁ (PGE₁), dimethylsulphoxide (DMSO), collagen type I (from bovine tendon acid-insoluble and human placenta acid-soluble), glutaraldehyde grade I and apyrase grade VII were from Sigma Chemical Co. (St. Louis, MO); electron microscope grade paraformaldehyde and plain polystyrene microspheres (10 μm) were from Polysciences Inc. (Warrington, PA); GPRP (Gly-Pro-Arg-Pro), a tetrapeptide known to inhibit fibrin polymerization and to prevent fibrinogen binding to platelets, PPACK, a thrombin specific anti-coagulant (D-phenylalanyl-L-prolyl-arginyl chloro-methyl ketone) and N-hydroxysuccinimido biotin (NHS-biotin) were from Calbiochem (La Jolla, CA.); purified human fibrinogen (high grade) was from American Diagnostica Inc. (Greenwich, CT); PAR₁ thrombin receptor activating peptide SFLLRN (Ser-Phe-Leu-Leu-Arg-Asn) was synthesized by the University of Toronto Peptide Synthesis Centre. Integrilin (Eptifibatide), the KGD (Lys-Gly-Asp) containing hepta-cyclopeptide that prevents fibrinogen binding to the GPIIb/IIIa receptor and blocks platelet aggregation, was kindly donated from COR Therapeutics (South San Francisco, CA); 0.8 μm polycarbonate filters were from Nucleopore (Cambridge, MA). Dulbecco’s phosphate buffer saline (PBS) was from Gibco/BRL Life Technologies (Ontario, Canada; containing 8 g/l NaCl, 2.16 g/l Na₂HPO₄, 0.2 g/l glucose, 0.2g/l KH₂PO₄); and sodium dodecyl sulfate (SDS), prestained standard molecular weight protein, Bio-safe Coomassie 250 blue stain, 2β-
mercaptoethanol and 7.5% acrylamide ready to go mini-gels were from Bio Rad (Richmond, CA). All other reagents were of analytical grade.

3.3.2 Antibodies

ReoPro (abciximab, chimeric Fab fragment of 7E3 monoclonal antibody), that inhibits binding of fibrinogen (and other adhesive ligands) to GPIIb/IIIa and prevents platelet aggregation was kindly donated by Centocor (Malvern, PA) [89,99]; sheep polyclonal affinity-purified anti-human fibrinogen antibody was from Cedarlane Labs Ltd (Ontario, Canada); R-phycoerythrin (PE)-conjugated neutravidin was from Molecular Probes Inc. (Eugene, OR); anti-collagen receptor monoclonal antibody specific for GPIa/Iia (clone Gi9) known to inhibit platelet adhesion to collagen was from Immunotech (Marseille, France); rabbit affinity-purified polyclonal anti-collagen type I antibody (raised against collagen type I from human and bovine placenta) was from Rockland (Gilbertsville, PA); and electron microscopy grade 20 nm gold conjugated streptavidin was from Zymed labs (San Francisco, CA).

3.3.3 Preparation of platelet-rich plasma and biotinylated washed platelets

Whole blood was collected without tourniquet from healthy donors (who were not taking medications for the last two weeks), after giving signed informed consent, into syringes pre-loaded with sodium citrate (0.38% final concentration), after discarding the first 2 ml. Whole blood was centrifuged at 120-150 xg for 5-10 min to prepare PRP [181]. Platelets in PRP were biotinylated where indicated by the addition of 10 μl (0.2 mg) per ml of PRP of biotin-NHS (in DMSO at 20 mg/ml) [146,213], and were then isolated in a three step washing procedure at room temperature [182]. Platelets were washed and resuspended in platelet
medium that contained 137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.4 mM NaH₂PO₄, 1 mM MgCl₂, 5.5 mM glucose and 0.35 % of albumin. The first wash was at pH 6.4 and included PGE₁ (1 µM final) and sodium citrate (0.38% final) [182]. The second and third washes were at pH 7.4. The final platelet medium was recalcified with 2 mM CaCl₂, contained apyrase (0.04 U/ml final) and was brought to a final platelet count of 3-3.5 x 10⁸/ml for platelet adhesion experiments and at 5 x 10⁸/ml for MP generation [182].

3.3.4 Flow cytometric assessment of washed platelets and MPs

Biotinylated washed platelets were assessed by flow cytometry (Beckton Dickinson, Mountain View, CA) with light scatter and fluorescence channels set on a logarithmic scale. Platelet specific events, including microparticles, were identified by gating on PE-conjugated neutravidin (PE-neutravidin) bound to biotinylated platelets and MPs. Single intact platelets were distinguished from microparticles by forward scatter size analysis. Ten thousand positive platelet specific events were analyzed and the mean fluorescence units per platelet and microparticle were quantitated. The gate was set to the immediate right of the background PE signal that the maximum number of microparticles, by definition smaller (based on forward scatter) than intact platelets, were analyzed. Purified microparticles were assessed as events smaller than the intact platelets based on forward scatter (to the left of the line) and were expressed as percent of total platelet specific events (96.6±1.0 % pure). Standard size beads set this scale at less than 0.8 µm.

In some instances, platelets were assessed by dual-labelled flow cytometry using platelet specific FITC conjugated anti-GPⅡb/IIIa, FITC conjugated anti-GPⅠb and PE conjugated anti-CD62 (P-selectin) monoclonal antibodies. Platelet washing procedure, monitored by flow cytometry using anti-GPⅡb/IIIa (or anti-GPⅠb) and/or anti-CD62 (P-selectin)
monoclonal antibodies, did not affect resting platelet condition prior to incubation with protein-coated and uncoated microspheres. Further, platelet biotinylation did not affect platelet function since no functional difference was noted between biotinylated or native WP, as assessed by flow cytometry (% MP generation, P-selectin expression or adhesion to protein coated or uncoated polystyrene microspheres (PCM/UPM)).

3.3.5 Generation and purification of platelet MPs

MPs were generated from biotinylated WP (5 x 10⁸/ml) upon stimulation with PAR1 thrombin receptor activating peptide SFLLRN (Ser-Phe-Leu-Leu-Arg-Asn, 100 μM) at 37°C for 20 min with gentle initial mixing. As assessed by flow cytometry, washed platelets generated 60.9±2.6 % MPs (n=5). MPs and platelets were then physically separated by an initial differential centrifugation (8000 xg for 15 minutes). The supernatant was then filtered through a 0.8 μm polycarbonate filter to exclude any remnant platelets. With this method, purified MPs were 96.6±1.0 % (n=5) pure as assessed by flow cytometry (Figure 17). Purified MPs were then used immediately in adhesion assay.
Figure 17 Washed platelets and MPs as assessed by flow cytometry

Resting washed platelets (panel 1) or platelets stimulated with 100 µM SFLLRN (SF) at 37 °C (panel 2) were assessed by flow cytometry. MPs, separated from platelets by differential centrifugation (8000 xg, 15 min) followed by filtration (0.8 µm) were 96.6±1.0 % (n=5) pure as assessed by flow cytometry (Panel 3).
3.3.6 Preparation of the adhesive protein coated microspheres

Polystyrene microspheres (10 µm) were degassed and washed in PBS prior to incubation for 1 hour at 37 °C with purified human fibrinogen (100 µg/ml in PBS), collagen type I from bovine tendon (100 µg/ml in PBS), collagen type I from human placenta (100 µg/ml in PBS) or albumin (100 µg/ml in PBS). The microspheres were then washed two times in PBS by a centrifugation at 350 xg for 5 min, were resuspended to the starting volume in PBS, were counted using hemocytometry (1.7-2.2 x 10^7/ml) and were then used immediately in the adhesion assay with either platelets or MPs suspended in final platelet medium containing 0.35 % albumin.

Surface fibrin monomers were generated directly on the fibrinogen-coated microspheres by cleaving surface-bound fibrinogen with human α-thrombin [136]. Briefly, fibrinogen-coated microspheres were mixed and incubated with 1.0 U/ml of human α-thrombin and 5 mM CaCl₂ for 10 min, at room temperature in the presence of 0.3 mM GPRP. Cleavage of fibrinogen was then arrested with 60 µM PPACK (thrombin-specific anticoagulant), and the microspheres were washed twice in PBS by centrifugation (350 xg for 5 min) and were resuspended in PBS. Fibrin-coated microspheres were then used immediately in the adhesion assay.

3.3.7 SDS-PAGE assessment of the surface immobilized ligands

The presence and purity of microsphere bound fibrinogen, thrombin-generated fibrin and collagen type I (bovine and human) were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions according to the methods of Laemmli [183]. Briefly, fibrinogen, fibrin and collagen type I-coated microspheres, prepared for the adhesion assay as described above, were incubated with SDS
under reducing (2β-mercaptoethanol) and non-reducing conditions for 10 min at 95 °C. Samples were loaded on 7.5% acrylamide mini-gels together with molecular weight standards. After electrophoresis, the gel was stained with Bio-safe Coomassie blue to visualize protein bands.

3.3.8 Microsphere-based adhesion assay

We developed a unique, microsphere-based, static flow cytometric adhesion assay to quantitate the surface adhesion of biotinylated/native platelets or purified MPs on polystyrene microspheres either uncoated (UPM) or protein coated with albumin, fibrinogen, fibrin or collagen type I (PCM) [214]. Briefly, 50 μl (15×10⁶) of biotinylated WP (suspended in final platelet medium containing 2 mM CaCl₂, 0.04 U/ml apyrase and 0.35 % albumin) were incubated with 10 μl (2×10⁵) of microspheres. In parallel, 100 μl of purified MPs (derived from the same platelet source, 96% pure) were incubated with 5 μl (10⁵) of plain and protein-coated microspheres. In all cases, plain and protein-coated microspheres (albumin, fibrinogen, fibrin, collagen type I, human and bovine) were assessed in parallel.

For inhibition studies, fibrinogen, fibrin and collagen type I-coated microspheres were pre-incubated (up to 30 minutes at 37 °C) with polyclonal antibodies to human fibrinogen (270 μg/ml) or collagen type I (125 μg/ml) prior to being used in the adhesion assay as described. In some cases, WP or MPs were preincubated with EDTA (10 mM), ReoPro (100 μg/ml), Integrilin (100 μg/ml) or 100 μg/ml of the GPIa/IIa specific monoclonal antibody (clone Gi9).

WP/MPs were then incubated with PCM/UPM at 37 °C for 20 min with occasional mixing, and were then washed gently twice (50 xg, 3 min) in 500 μl Hepes Tyrodes buffer (HTB containing: 137 mM NaCl, 3.7 mM KCl, 16 mM NaHCO₃, 5 mM MgCl₂, 3.5 mM
Hepes, 5.5 mM glucose, 0.2 % bovine albumin; pH 7.4) and incubated with a saturating concentrations of PE-neutravidin for 20 min, at room temperature. Finally, samples were then diluted in HTB, fixed in 1 % fresh paraformaldehyde and were analyzed by flow cytometry (Figure 18).

3.3.9 Flow cytometric assessment of platelet/MP adhesion

Samples were analyzed on a Beckton Dickinson FACScan flow cytometer (Mountain View, CA) with CELLQuest software. Microspheres from the adhesion assay were identified and assessed by flow cytometry based on their forward (mean size) and side scatter (complexity or density of beads) characteristics. 1000 microspheres were analyzed per experiment and the average fluorescence intensity per bead was recorded. Background fluorescence of microspheres was determined by analyzing microspheres incubated in the presence of fluorochrome (PE-neutravidin) but in the absence of platelets or MPs. The average fluorescence signals (arbitrary units) of microspheres incubated with washed platelets or MPs were then corrected for non-specific fluorochrome binding to microspheres in all determinations.
Figure 18 Flow cytometric analysis of WP/MP adhesion to albumin or fibrinogen-coated microspheres

Flow cytometric dot plots of washed platelets (Panel 1a) or MPs (Panel 2a) incubated with fibrinogen or albumin-coated microspheres (R1) are shown. The microsphere forward scatter histogram (Panel 1b) was gated on a single bead (R2) and adherent platelets or MPs were quantified by platelet specific fluorescence signal per bead (Panels 1c and 2b). Panel 1c shows histograms of washed platelets adherent to fibrinogen and albumin-coated microspheres. Panel 2b shows overlayed histograms of MPs adherent to fibrinogen and albumin-coated microspheres.
3.3.10 Scanning electron microscopy assessment of platelet/MP adhesion to protein-coated and uncoated microspheres

In parallel with the flow cytometric experiments, aliquots of protein-coated and uncoated microspheres incubated with WP or MPs were prepared for scanning electron microscopy and immunogold SEM. Briefly, 25 μl aliquots of samples from the immunoadhesion flow cytometric assays were washed twice in 300 μl of PBS with a gentle centrifugation (50 xg, 5 min) and were then fixed in 2.5% glutaraldehyde for SEM assessment. Samples were then dehydrated through a gradient ethanol series and critical point dried with CO₂ [215]. Samples were then coated with gold to render them electroconductive using a Polaron Sputter Coater for 120 seconds at 100 mA (Polaron Equipment Ltd., Watford, England). Finally, samples were imaged with a Hitachi S-570 electron microscope at the 20 kV accelerating voltage, at the Faculty of Medicine, University of Toronto (Toronto, Canada).

For immunogold SEM studies, microsphere-bound biotinylated microparticles were identified with 20 nm gold particles conjugated with streptavidin [216]. Briefly, 20 μl aliquots from adhesion assays were washed in PBS (50 xg, 5 min) and were then incubated with gold-streptavidin (20 nm colloidal gold particles, final dilution 1:20 in PBS) for 30 min at 37°C. After incubation, unbound gold-streptavidin was removed with a gentle centrifugation of the beads at 50 xg for 5 min after which they were fixed with 4% paraformaldehyde. After graded ethanol dehydration and critical point drying, samples were carbon coated using a carbon coater “Edwards”, High Vacuum Ltd (Crawley, Sussex, U.K.). Gold particles associated with biotin-labelled MPs were localized by back-scattered electron imaging at a 20 kV accelerating voltage using a Hitachi S-570 electron microscope at the Faculty of Medicine, University of Toronto (Toronto, Canada) [217].
3.3.11 Statistics

Statistical analysis was performed with InStat 3.01 PC software (Graph Pad Software, San Diego, CA). Results were expressed as mean values of up to 4-7 experiments ± standard error of the mean (SEM). The means of three or more groups were compared by one-way analysis of variance (ANOVA) followed by Tukey or Student-Newman-Keuls multiple comparisons post-test to determine which groups are significantly different from other. A value of p<0.05 was accepted as significant.
3.4 Results

3.4.1 Platelet adhesion

We assessed and compared the adhesion of platelets and MPs to surface-bound adhesive ligands. Unactivated, biotinylated washed resting platelets were resuspended with protein coated or uncoated microspheres and platelet adhesion was assessed by flow cytometry and visualized by SEM. As platelet adhesion to surface immobilized fibrinogen, fibrin and collagen has previously been demonstrated to be metal-ion dependent, EDTA samples were included as controls [10,25]. The effects of a series of inhibitors (ReoPro, Integrilin, anti-GPIIb/IIIa monoclonal antibody and anti-collagen or fibrinogen polyclonal antibodies) on washed platelets adhesion to microspheres were also assessed. Results are expressed in arbitrary fluorescence units recorded per bead (Fl.U.). Further, the results of inhibition of platelet adhesion to PCM/UPM are expressed as percent of total inhibition of WP adhesion calculated relative to the fluorescence signal of albumin-coated microspheres in the presence of EDTA.

Protein ligand purity and ligand presence on PCM was verified by SDS-PAGE. Surface-bound ligands fibrinogen, fibrin and collagen type I were judged to be free of contaminating proteins and present on the beads, as assessed by SDS-PAGE. Fibrinogen, eluted from the beads, was visualized as a single band (340 kDa) under non-reducing conditions, but as Aα, Bβ and γ-polypeptide chains (63, 56 and 47 kDa, respectively) under the reducing conditions [9]. Fibrin, eluted from the beads, was visualized as a single non-reduced band (333.2 kDa), and as Aα, Bβ and γ-chains under reducing conditions. Notably, while the fibrin-derived γ-chain was the same size as that of fibrinogen, the Aα and Bβ-chains were of a slightly lower molecular weight than those of fibrinogen Aα and Bβ chains, due to thrombin cleavage.
Collagen type I, eluted from beads was visualized as α1 and α2 chains under reduced conditions, each around 130 kDa (Figure 19b) [9].

Microsphere surface protein coating and integrity was assessed visually by SEM. Compared to the plain polystyrene surface, a protein coating (albumin, fibrinogen, fibrin, collagen type I-bovine and human) was evident by visual change in the form of abundant layer of protein material over the microspheres seen as “halo” effect on SEM images (Figure 20) [215]. Bovine, acid-nonsoluble collagen type I was detected as distinct fibrils on the microsphere surface, in contrast to the smooth coating produced by human, acid-soluble collagen type I. X-ray photoelectron-spectroscopy (XPS) [218] or X-ray crystalography [219,220] are methods, used by others, to demonstrate and characterize full protein adsorption to biomaterial surfaces under conditions similar to our methods.
Figure 19a SDS-PAGE analysis of fibrinogen and fibrin

Fibrinogen (Ia, IIa) and fibrin (Ib, IIb) were eluted from coated microspheres in SDS and analyzed by SDS-PAGE under non-reduced (I) and reduced (II) conditions and Coomassie blue stained. Fibrinogen and fibrin are both visualized as single bands under non-reduced conditions, and as distinct α, β and γ-chains under reducing conditions.
Collagen type I was eluted from coated microspheres in SDS and analyzed by SDS-PAGE under reduced conditions and stained with Coomassie blue. Approximately 130 kDa \( \alpha 1 \) and \( \alpha 2 \) chain bands were visualized under reducing conditions.
Figure 20 Scanning electron micrographs of protein-coated and uncoated microspheres

Plain uncoated, and albumin, fibrinogen, fibrin, collagen 1 (bovine) and collagen 2 (human) coated microspheres were fixed in 2.5 % glutaraldehyde and upon standard preparation procedure (ethanol graded dehydration and critical point drying) examined by scanning electron microscopy.
3.4.1.1 Washed platelet adhesion to albumin-coated microspheres

Albumin was chosen as the control ligand adhesion surface, as we and others have shown that neither WP or MPs adhere to albumin-coated microspheres [219]. As assessed by flow cytometry, albumin-coated microspheres incubated with washed resting platelets, demonstrated a low average fluorescence signal consistent with a lack of adherent platelets, both in the absence (Fl.U.=17.4±1.7; n=7; Figure 21a) and presence (Fl.U.=16.5±2.5; n=7; Figure 21b) of EDTA. Consistent with this, SEM images of WP incubated with albumin-coated microspheres revealed only very rare platelets that had lost the resting, discoid shape and had acquired a spherical shape with rare protruding pseudopodias extended towards the microspheres surface. This morphology is consistent with an early state of platelet activation without spreading and platelet adhesion (Figure 22). Thus we found, rare or no platelets on albumin-coated microsphere as seen on SEM (Figure 22).

3.4.1.2 Washed platelet adhesion to plain microspheres

Uncoated polystyrene microspheres (UPM) incubated with resting WP demonstrated low fluorescence, indicating a low level of platelet adhesion to the native bead surface (Fl.U.=32.9±6.4; n=7) (Figures 21a and 21b).

Similar to what had been observed with albumin PCM, SEM images of resting WP incubated with plain microspheres revealed occasional platelets that had lost their resting discoid shape, and had acquired a spherical shape with protruding pseudopodia extended towards the microsphere surface, suggestive of an early state of platelet activation without platelet spreading and adhesion (Figure 22). Consistent with the flow cytometric results, more platelets were visualized by SEM on the plain polystyrene surface, than were seen on albumin-coated microspheres as visualized by SEM (Figure 22). In addition, similar to what
was observed with albumin PCM, WP adhesion to UPM was not affected by the addition of EDTA (34.9±8.9; n=7) (Figure 21b).
Figure 21a Washed platelet adhesion to protein-coated or uncoated microsphere as assessed by flow cytometry

Plain, and albumin, fibrinogen, fibrin, collagen type I (bovine (1) and human (2)) coated polystyrene microspheres were incubated with washed biotinylated resting platelets, then with neutravidin-PE and were assessed by flow cytometry for evidence of platelet adhesion. Platelet adhesion is expressed in arbitrary average fluorescence units per bead with standard mean error (mean Fl. U.±SEM; n=4-7; p<0.001*, see text pg. 122).
Figure 21b Inhibition studies of platelet adhesion to protein-coated and uncoated microspheres by flow cytometry

Washed resting biotinylated platelets were incubated with PCM or UPM and with one of EDTA (10 mM), ReoPro (100 µg/ml), Integrilin (100 µg/ml), polyclonal anti-fibrinogen antibody (polycl anti-fgn; 270 µg/ml), polyclonal anti-collagen type I antibody (polycl anti-coll; 125 µg/ml), anti-GPIa/IIa monoclonal antibody (100 µg/ml) and surface bound platelets were then quantified by flow cytometry. Platelet adhesion is expressed in arbitrary units of average fluorescence intensity per bead with standard error of the mean (mean Fl. U.±SEM; n=3-7).
Figure 22 Scanning electron micrographs of washed platelets adherent to protein-coated and uncoated surfaces

Plain and albumin, fibrinogen, fibrin or collagen type I (bovine) coated microspheres were incubated with resting, washed, biotinylated platelets for 20 min at 37 °C, washed, fixed with 2.5% glutaraldehyde and upon standard preparation procedure, visualized by SEM.
3.4.1.3 Washed platelet adhesion to fibrinogen-coated microspheres

In contrast to the low levels of adhesion to albumin-coated microspheres, WP bound avidly to fibrinogen-coated surfaces (Fl. U.=509.6±82; n=6; p<0.001 by ANOVA) (Figure 21a). Consistent with this high level of fluorescence, SEM revealed visually that fibrinogen-coated microspheres were covered with layers of spread, "fried-egg shaped", visually apparently activated platelets with extending pseudopodia (Figure 22). Thus, contact with immobilized fibrinogen is sufficient for platelet activation, since resting washed platelets were incubated with fibrinogen-coated microspheres.

We next probed the mechanism of platelet adhesion to immobilized fibrinogen with EDTA, GPIIb/IIIa antagonists (ReoPro and Integrilin) and with polyclonal anti-fibrinogen antibodies. As expected, platelet adhesion was metal-ion dependent as 10 mM EDTA almost completely eliminated platelet binding (94.5%±1.9 inhibition; n=6; Figure 21b; Table 1a). Adhesion was also GPIIb/IIIa dependent, with 92.8%±2.3 inhibition by ReoPro (n=6) and 94.3%±1.5 inhibition by Integrilin (n=6; Figure 21b; Table 1a). Consistent with this, preincubation of the fibrinogen-coated microspheres with a polyclonal anti-fibrinogen antibodies inhibited platelet adhesion by 96.0%±1.7 (n=3; Figure 21b; Table 1a), confirming the fibrinogen specificity of platelet adhesion to microspheres in this system.

3.4.1.4 Washed platelet adhesion to fibrin-coated microspheres

Washed resting platelets adhered to fibrin-coated microspheres to a slightly lesser degree than to their fibrinogen counterparts (Fl.U.=407.3±75.3; n=6; Figure 21a) but this difference was not statistically significant (p>0.05, by ANOVA). These observations were confirmed visually by SEM (Figure 22). Platelets adherent to the fibrin-coated surfaces appeared completely flattened ("pancaked"), indicating complete platelet activation with spreading [9].
Full platelet activation was induced upon contact with immobilized fibrin, as resting washed platelets had been added to the fibrin-coated microspheres in the absence of a platelet agonist. Additionally, small membrane particles, presumed to be MPs, were seen to be "budding off" from the platelet membrane surface, forming aggregates and adhering to the platelet or bead surface (Figure 23).

Platelet adhesion to fibrin, like that to fibrinogen, was metal-ion dependent as EDTA abolished platelet adhesion to the level of the EDTA/albumin control (90.0%±5 inhibition; n=6) (Figure 21b; Table 1a). Platelet adhesion to fibrin, similarly involved the integrin GPIIb/IIIa, since adhesion was inhibited by 92.2%±2.5 by ReoPro (n=5) and by 93.0%±1.8 with Integrilin (n=5), two GPIIb/IIIa blocking agents (Figure 21b; Table 1a) [12,94,96]. In the presence of polyclonal anti-fibrinogen antibodies that is known to cross-react with fibrin, platelet adhesion to fibrin-coated microspheres was inhibited by 94.3%±1.2 (n=3), in a manner similar to the inhibition of platelet adhesion to fibrinogen-coated microspheres (Figure 21b; Table 1a).

Table 1a Inhibition studies of platelet adhesion to fibrinogen and fibrin-coated microspheres

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<th>Conditions</th>
<th>Protein-coated microspheres</th>
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<tr>
<td></td>
<td>ALBUMIN(%inh)</td>
<td>FIBRINOGEN(%inh)</td>
<td>FIBRIN(%inh)</td>
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<tr>
<td>EDTA</td>
<td>100</td>
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<tr>
<td>Polycl anti-fgn</td>
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<td>96.0±1.7</td>
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n=3-6 (mean±SEM);
Polyclonal anti-fibrinogen antibodies (Polycl anti-fgn)

Results are expressed as % inhibition relative to platelet adhesion to albumin-coated microsphere in the presence of EDTA (10 mM).
Fibrin-coated microspheres were incubated with resting washed biotinylated platelets for 20 min at 37 °C, washed, fixed with 2.5% glutaraldehyde and upon standard preparation procedure, visualized by SEM. Two different magnifications (20-40K) of the membrane surface particles generated from fully spread adherent platelets on fibrin-coated microspheres are shown.
3.4.1.5 Washed platelet adhesion to collagen type I-coated microspheres

Washed resting platelets adhered to both bovine and human collagen type I coated surfaces, (Fl.U.=260.3±46.1; n=4) and (Fl.U.=252.0±55.2; n=5), respectively. Notably, however, platelet adhesion to collagen CM was considerably less than that to microspheres coated with fibrinogen (2 fold higher) or fibrin (1.6 fold higher) (Figure 21a). SEM studies confirmed visually that platelets were indeed adherent to the collagen-coated microspheres (Figure 22), supporting the flow cytometric findings (Figure 21a) and indicating that immobilized collagen could induce contact platelet activation. Surface-bound platelets were activated, spread and “balloon like” with visible “bleb” like structures that may represent microparticles emanating from the platelet membrane surface (Figure 22). Adherent small particles, seen adjacent to the platelets are presumed to be MPs shed from the platelets upon contact activation, that were then bound back to the collagen-coated surface (Figure 22).

As predicted, washed platelet adhesion to collagen type I was cation-dependent, being abolished in the presence of EDTA for both bovine (82.0±1.7 inhibition; n=3) and human (76.3±5.8 inhibition; n=3) collagen type I coated microspheres (Figure 21b; Table 1b) [24]. Platelet adhesion to collagen type I was also abolished by an anti-GPIa/IIa monoclonal antibody (clone Gi9) that binds to the collagen receptor on the platelet membrane surface (bovine 86.3±2.4; n=3 and human 86.0±3.1 inhibition, n=3) [61,103]. Finally, preincubation of collagen-coated microspheres with a polyclonal anti-collagen antibody inhibited washed platelet adhesion to bovine collagen by 85.0±6.0 (n=3) and to human collagen by 88±8.3 (n=3), indicating specificity of the ligand binding interaction in these experiments (Figure 21b; Table 1b).
Table 1b Inhibition studies of platelet adhesion to immobilized collagen type I (bovine and human)

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<tr>
<th>Conditions</th>
<th>Protein-coated microspheres</th>
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<td>ALBUMIN (% inh.)</td>
<td>BOVINE (% inh.)</td>
<td>HUMAN (% inh.)</td>
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<td>EDTA</td>
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<td>76.3±5.6</td>
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<tr>
<td>GPIa/IIa mab (Gi9)</td>
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<td>86.3±2.4</td>
<td>86.0±3.1</td>
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<tr>
<td>Polycl anti-coll ab</td>
<td></td>
<td>85.0±6.0</td>
<td>87.8±8.3</td>
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n=3 (mean±SEM);
Monoclonal antibody against collagen receptor GPIa/IIa clone Gi9 (GPIa/IIa mab (Gi9));
Polyclonal anti-collagen antibody (Polycl anti-coll ab).

Results are expressed as % inhibition relative to platelet adhesion to albumin-coated microsphere in the presence of EDTA (10 mM).
3.4.1.6 Contact platelet activation

Static, adhesion, microsphere-based flow cytometric assessment of washed platelet adhesion to immobilized vascular ligands involved the incubation of resting platelets with PCM/UPM. The resting condition of the washed platelets was confirmed flow cytometrically by assessing platelet activation markers prior to incubation with PCM/UPM. Platelet activation markers include the generation of MPs (increased upon activation), up-regulation of platelet GPIIb/IIIa β3 integrin and of P-selectin, and the down-regulation of GPIb [17,49,107,149].

Platelet washing and biotinylation did not result in activation as MPs were not generated, GPIIb/IIIa was not up-regulated (or GPIb was not down-regulated) and α-granule P-selectin was not expressed, when compared to resting and SFLLRN fully activated platelets (Table 1c). Upon washed platelet incubation with PCM/UPM, and in the absence of any added agonist, platelet activation was observed, as determined by the increased generation of MPs (%), GPIIb/IIIa up-regulation (or GPIb down-regulation) and P-selectin expression in the bulk of free, unbound platelets (Table 1c).

SEM studies of microspheres confirmed visually a state of platelet activation that varied among the different ligands immobilized on the polystyrene surface: Albumin and polystyrene surfaces were the least platelet reactive, and resulted in only an early state of platelet activation, without adhesion. Immobilized fibrinogen, fibrin and collagen type I however, induced full platelet activation, spreading and adhesion, as visualized by SEM (Figures 22 and 23). Furthermore, fibrin(ogen) and collagen-coated surfaces appeared to generate, platelet membrane surface particles, that aggregated among themselves, and adhered to the collagen or fibrin-coated surface or to adjacent platelets (Figures 22 and 23). We presume that these “blebs” are MPs generated upon contact platelet activation since they
are only seen on fibrin(ogen)/collagen-coated microspheres and since we demonstrated the
generation of MPs in the bulk, after platelet incubation with protein-coated and uncoated
microspheres.

Table 1c Activation of non-adherent platelets after incubation with protein-coated and
uncoated microspheres

<table>
<thead>
<tr>
<th></th>
<th>% MPs</th>
<th>GPIb FL.U.</th>
<th>GPIIb/IIIa FL.U.</th>
<th>P-sel FL.U.</th>
<th>P-sel % pos (M1)</th>
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<td>REST PRP</td>
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<td>WP+SF</td>
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<td>99.31</td>
<td>419.15</td>
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<td>35.4</td>
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<td>WP+PLAIN M</td>
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<td>112.68</td>
<td>293.46</td>
<td>15.23</td>
<td>48.27</td>
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<td>WP+FIBRINOGEN CM</td>
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<td>93.91</td>
<td>384.63</td>
<td>97.25</td>
<td>96.29</td>
</tr>
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<td>100.48</td>
<td>374.85</td>
<td>106.19</td>
<td>96.91</td>
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<td>WP+COLLAGEN (B) CM</td>
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<td>384.10</td>
<td>97.09</td>
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<td>WP+COLLAGEN (H) CM</td>
<td>12.17</td>
<td>98.45</td>
<td>369.12</td>
<td>98.49</td>
<td>95.69</td>
</tr>
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</table>

Table 1c is a summary of representative experiment of platelet activation and MP
formation upon the incubation of resting platelets with PCM/UPM, as assessed by flow
cytometry. The % MPs generated, and the expression of GPIb, GPIIb/IIIa and P-selectin
(FL.U.) and % of P-selectin positive events (M1)) by washed platelets (WP) incubated with
protein-coated microspheres (albumin, fibrinogen, fibrin, collagen bovine (B) and human (H)
CM) or uncoated plain microspheres (plain M) were compared to resting PRP (rest PRP),
resting biotinylated WP (rest WP) or WP stimulated with 100 μM of SFLLRN (WP+SF).
3.4.1.7 Comparison of platelet adhesion to protein-coated and uncoated microspheres

We compared the adhesion of washed platelets to immobilized fibrinogen, fibrin and collagen type I (bovine and human), relative to platelet binding on albumin-coated microspheres, to define our static adhesion model of platelet adherence to surface immobilized ligands, for subsequent comparison of MP adhesion under identical conditions.

As shown in Figure 24, platelet adhesion was greatest to immobilized fibrinogen, with a 32 fold increased binding relative to albumin (32.3±7.4X; n=6; p<0.001 by ANOVA, followed by Student-Newman-Keuls multiple comparisons post-test), followed by fibrin (24.3±4.7X; n=6; p<0.001), collagen type I bovine (12.9±1.6X; n=4; p<0.01), collagen type I human (13.9±2.1X; n=5; p<0.01) and plain polystyrene microspheres (1.8±0.2X; n=7; p>0.05). Platelet adhesion to immobilized fibrinogen and fibrin were not statistically different (p>0.05, by ANOVA). Similarly, there was also no statistical difference in the adhesion of platelets to bovine or human collagen type I (p>0.05). However, adhesion to collagen was significantly lower than to fibrinogen or fibrin (p<0.01, by ANOVA).

SEM studies confirmed visually all of these differences (Figure 22). All ligands supported contact platelet activation in the absence of a platelet agonist. Albumin-coated and plain polystyrene surfaces did not result in the generation of adherent and spread platelets (Figure 22).
Figure 24 Washed platelet adhesion to protein-coated microspheres, relative to platelet adhesion to albumin-coated microspheres as assessed by flow cytometry

Plain and albumin, fibrinogen, fibrin, and collagen type I (bovine (1) and human (2) coated microspheres were incubated with resting washed platelets and evaluated by flow cytometry for evidence of platelet adhesion. Platelet adhesion to protein-coated microspheres was compared to that of albumin coated control (n=4-7±SEM; p>0.05*; p<0.001**; p<0.01***, see text).
3.4.2 MP adhesion

The ability of purified biotinylated platelet microparticles (MPs) to bind to the surface immobilized vascular ligands fibrinogen, fibrin and collagen type I and to the control protein albumin, was assessed in the static flow cytometric adhesion assay and compared to washed platelet adhesion. All flow-cytometric data were corrected for the background fluorescence signal obtained by the incubation of the fluorochrome (PE-neutravidin) and the PCM/UPM in the absence of platelets or of particles. Flow cytometry data were supported by SEM studies, while immunogold SEM studies verified the platelet origin of the MPs found on the protein-coated microspheres. In addition, the mechanism(s) of MP adhesion to immobilized ligands was compared with the binding of platelets to ligand-coated microspheres.

3.4.2.1 MP adhesion to albumin-coated microspheres

We have shown by flow cytometry and SEM that washed platelets do not adhere to albumin-coated microspheres (Figures 21a and 22). MPs are similarly unable to adhere to albumin-coated microspheres. Albumin-coated control beads demonstrated a low fluorescence signal consistent with a lack of MP adhesion (Fl.U.=22.81±4.83; n=4) (Figure 25a). Consistent with this, scanning electron micrographs of MPs incubated with albumin-coated microspheres revealed only sporadic, spherical, single MPs associated with the surface (2-3 per 1 μm²) (Figure 26 and 27).

As assessed by flow cytometry, MP binding to albumin-coated microspheres was not affected by the addition of EDTA (Fl.U.=23.44±3.04; n=4) (Figure 25b). SEM of EDTA treated beads was similarly no different from that of MPs incubated with albumin-coated microspheres alone, supporting the flow-cytometric observation (Figure 26).
Figure 25a MP adhesion to fibrinogen, fibrin and collagen type I (bovine (1) and human (2)) coated microspheres assessed by flow cytometry

Purified biotinylated MPs were incubated with protein-coated microspheres, then with secondary fluorochrome and were assessed by flow cytometry for evidence of adhesion. Results are expressed in arbitrary fluorescence units per bead with standard error of the mean (mean Fl.U.±SEM; n=4; p<0.001*, see text pg. 141).
Figure 25b Inhibition studies of MP adhesion to protein-coated and uncoated microspheres assessed by flow cytometry

PCM/UPM were incubated with EDTA (10 mM), ReoPro (100 μg/ml), Integrilin (100 μg/ml), anti-GPIa/IIa monoclonal antibody (100 μg/ml), polyclonal anti-fibrinogen antibody (polycl anti-fgn; 270 μg/ml), polyclonal anti-collagen type I antibody (polycl anti-coll; 125 μg/ml) and in the presence and absence of biotinylated MPs. MP adhesion was then assessed by flow cytometry. The results are presented in arbitrary units of average fluorescence intensity with standard mean error (mean Fl.U.±SEM; n=4; see text).
Figure 26 Scanning electron micrographs of MPs adherent to albumin coated and uncoated plain microspheres

Photomicrographs of albumin-coated and plain polystyrene uncoated microspheres incubated with or without EDTA (10 mM) in the presence of MPs, for 20 min at 37 °C. Samples were washed, fixed with 2.5% glutaraldehyde and upon standard preparation procedure, visualized by scanning electron microscopy. Presented are representative photomicrographs from n=3 experiments.
Figure 27 Scanning electron micrographs of MPs adherent to protein-coated and uncoated microspheres:

Representative photomicrographs of purified, biotinylated MPs that were incubated with protein-coated or uncoated microspheres are shown. Microspheres and MPs were incubated for 20 min at 37 °C, washed, fixed with 2.5% glutaraldehyde, dehydrated and visualized by SEM (n=3). Abbreviations: Collagen (coll).
3.4.2.2 MP adhesion to plain polystyrene microspheres

As measured by flow cytometry, MP adhesion to plain polystyrene microspheres (UPM), (Fl.U. = 28.77±5.8; n=4), was not statistically different (p<0.05 by ANOVA; Figure 25a) from adhesion to albumin-coated surfaces. SEM studies, like those of albumin surfaces, revealed single, sporadic MPs measuring 0.02-0.2 μm on the surface. Manual counting of surface-bound sporadic MPs on three blindly taken squares (1 μm²), at 20xK or 40xK magnification of SEM photomicrographs, revealed slightly more MPs on polystyrene surface than on albumin (6-8 per1 μm²). Little difference in MP adhesion to UPM was noted in the presence of 10 mM EDTA by either flow cytometry (Fl.U. = 26.57±5.7; n=4) (Figure 25b) or SEM analyses (Figure 26).

In immunogold SEM studies, sporadic biotinylated particles, labelled with streptavidin-gold were observed with reverse polarity scanning consistent with rare MPs associated with the uncoated polystyrene surface. More gold particles associated with MPs were observed on plain polystyrene than on the albumin coated surface supporting the SEM studies and confirming that the MPs originated from biotinylated washed platelets. Streptavidin-gold binding was not observed on an albumin-coated surface incubated with streptavidin-gold in the absence of added MPs (data not shown).

3.4.2.3 MP adhesion to fibrinogen-coated microspheres

In contrast to MP adhesion to uncoated or albumin-coated surfaces, large numbers of MPs were adherent to fibrinogen-coated microspheres, as measured by flow cytometry (Fl.U. = 94.1±9.1; n=4) (Figure 25a). When compared to albumin-coated microspheres, MP adhesion to immobilized fibrinogen was significantly increased (4 fold; p<0.01 by ANOVA). SEM images confirmed visually an abundance of MPs on immobilized fibrinogen surfaces in
the form of singular (size=0.02-0.2 μm) or clusters of MPs (size=0.2-0.6 μm; Figure 27). In addition, rare, large aggregates or grape-like clusters up to 1 μm were seen. The relative numbers of MPs on the immobilized fibrinogen surface was determined by counting MPs on three blindly taken squares (1 μm²) at 20xK or 40xK magnification of SEM photomicrographs. If it was possible, the relative number of MPs forming an aggregate were counted, too. There were 18-23 MPs per 1 μm² on fibrinogen CM which was 9-12 fold greater than the numbers found on albumin-coated surfaces.

MP adhesion to immobilized fibrinogen was completely inhibited (99.0%±1.0 inhibition; n=3) in the presence of EDTA to the level of the control albumin-coated surface, indicating metal-ion dependency of MP binding to immobilized fibrinogen. Co-incubation of fibrinogen-coated microspheres (CM) with the GPIIb/IIIa blocking agents ReoPro and Integrilin inhibited MP adhesion by 99.6%±0.3 and 95.3%±2.3 respectively (n=3), to the level of the EDTA control (Figure 25b; Table 2a). ReoPro and Integrilin are specific for GPIIb/IIIa and are known to block fibrinogen binding to washed platelets [99,189] as confirmed in the platelet adhesion experiments described above (Figure 21b). These observations indicate that GPIIb/IIIa is the functional MP receptor for static adhesion to surface-bound fibrinogen. In addition, preincubation of fibrinogen-coated microspheres with polyclonal anti-fibrinogen antibody inhibited MP adhesion to fibrinogen by 94.0%±2.64 (n=3), confirming fibrinogen specificity of MP adhesion to beads in this system (Figure 25b, Table 2a).

Scanning electron micrographs of EDTA, ReoPro, Integrilin and polyclonal anti-fibrinogen antibody-treated MPs/fibrinogen-coated microspheres confirmed visually the flow cytometric observations (Figures 28). MP adhesion and clustering of aggregated MPs were
completely abolished in these conditions to the level of the albumin control (0-3 MPs per 1 μm²) as observed by SEM (Figure 28).

Immunogold SEM studies did not reveal non-specific binding of gold particles to albumin-coated microspheres and only sporadic gold particles were detected upon incubation of biotinylated MPs with albumin-coated microspheres, that correlated with the secondary images of rare MPs bound to the albumin surface (Figure 29). In sharp contrast, abundant streptavidin-gold labelled singular and clustered biotinylated MPs, were observed by reverse polarity immunogold-scanning electron microscopy of MP/fibrinogen-coated microspheres, that correlated with the secondary MPs images and were consistent with the singular and clustered MPs observed by SEM (Figure 29).

These observations support our hypothesis that MPs adhere to immobilized fibrinogen through mechanism(s) similar to that of platelet adhesion. MP adhesion to fibrinogen was metal-ion dependent, mediated by functional GPIIb/IIIa receptors and fibrinogen specific. Furthermore, a previously unknown ability of MPs to aggregate among themselves, as platelets do, has revealed by SEM and immunogold-SEM images (Figures 27, 28 and 29). Surface-bound purified, biotinylated MPs were confirmed to be platelet-derived by streptavidin-gold identification of singular and clustered MPs on immobilized fibrinogen.
Figure 28 Scanning electron micrographs of MPs adherent to fibrinogen-coated microspheres.

Representative photomicrographs of fibrinogen-coated microspheres incubated with EDTA (10 mM), ReoPro (100 μg/ml), Integriin (100 μg/ml) and polyclonal affinity-purified anti-fibrinogen antibody (polycl anti-fgn ab; 270 μg/ml) in the presence and absence of MPs for 20 min at 37 °C. Samples were washed, fixed with 2.5% glutaraldehyde and upon standard preparation procedure, visualized by SEM (n=3).
Figure 29 Immunogold scanning electron micrographs of surface-bound MPs

Protein-coated microspheres were incubated with purified, biotinylated MPs, washed, immunolabelled with streptavidin-gold, washed, fixed with paraformaldehyde and upon standard preparation procedure, carbon coated and visualized by back-scatter imaging (n=3). Back-scattered images of MP binding to albumin, fibrinogen, fibrin and collagen type I coated microspheres and the corresponding secondary images are shown.
3.4.2.4 MP adhesion to fibrin-coated microspheres

As shown on Figure 25a, as assessed by flow cytometry, MP adhesion was much greater to fibrin CM than to microspheres coated with other ligands or with albumin, (Fl.U.=180.3±13.7; n=4). MP adhesion to immobilized fibrin was 9 fold greater than that to MPs bound to albumin CM. This difference was highly statistically significant (p<0.001 by ANOVA). Abundant MP adhesion to surface immobilized fibrin was confirmed visually by SEM. MPs on fibrin CM were singular, spherical or “flattened, spread”, 0.05-0.2 μm in size or they formed clusters of aggregated MPs (0.2-0.8 μm in size) (Figure 27 and 30). MPs on fibrin CM were quantified from three blindly taken squares of 1 μm² from SEM photomicrographs taken at 20 or 40 K magnification. 28-37 MPs per μm² were observed on fibrin-coated microspheres that was 14 to 18 fold greater than on albumin CM. MPs appeared morphologically different on immobilized fibrin than on fibrinogen. MPs were more flattened and spread, similar to the appearance of platelets adherent to this surface immobilized ligand (Figure 27 and 30).

MP adhesion to fibrin-coated microspheres was metal-ion dependent, being completely abolished in the presence of EDTA (96.5%±2.1;n=3; Figure 25b; Table 2a). MP adhesion to immobilized fibrin was also GPIIb/IIIa dependent, with 95.7%±1.8 inhibition by ReoPro (n=4) and with 92.7%±3.8 inhibition by Integrilin (n=3; Figure 25b; Table 2a) [12,94,96]. In addition, incubation of fibrin-coated microspheres with polyclonal anti-fibrinogen antibodies fully inhibited MP adhesion to immobilized fibrin (96%±1.8; n=3; Figure 25b; Table 2a).

The inhibition of MP adhesion and loss of the observed clusters of aggregated MPs on fibrin-coated microspheres in the presence of EDTA, ReoPro, Integrilin and polyclonal anti-fibrinogen antibody was confirmed visually with SEM and fully supported the flow
cytometric results (Figure 30). MP adhesion and clusters of aggregated MPs were abolished by these agents to the level of the albumin control (2-3 MPs per 1 μm²), as observed by SEM (Figure 30).

Immunogold studies confirmed the origin of platelet-derived microparticles. An abundance of singular and clustered gold particles correlated with singular and aggregated MPs observed on both secondary images by reverse polarity SEM and common SEM images (Figure 29).

These findings confirm our hypothesis that MPs adhere to fibrin in a manner similar to that of platelets. However, MP adhesion to immobilized fibrin was greater than that to fibrinogen, in contrast what was observed for platelets, although similar mechanisms were involved. MP adhesion to fibrin CM was metal-ion dependent, mediated by the GPIIb/IIIa receptor and was fibrin specific, as was platelet adhesion to surface immobilized fibrin [12,96]. MPs aggregated on this surface as observed by SEM and immunogold SEM images (Figures 27, 29 and 30). Surface-bound purified, biotinylated MPs were confirmed to be platelet-derived by streptavidin-gold identification of singular and clustered MPs on immobilized fibrin.
Table 2a Inhibition studies of MP adhesion to immobilized fibrinogen and fibrin

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<th>Conditions</th>
<th>ALBUMIN (% inh)</th>
<th>FIBRINOGEN (% inh)</th>
<th>FIBRIN (% inh)</th>
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n=3-4 (mean±SEM);
Polyclonal anti-fibrinogen antibody (polycl antifgn ab)

Results are expressed as % inhibition relative to MP adhesion to albumin-coated microsphere in the presence of EDTA (10 mM).
Figure 30 Scanning electron micrographs of MPs adherent to fibrin-coated microspheres

Representative photomicrographs of fibrin-coated microspheres incubated with EDTA (10 mM), ReoPro (100 μg/ml), Integrilin (100 μg/ml) and polyclonal affinity-purified anti-fibrinogen antibody (polycl anti-fgn ab; 270 μg/ml) in the presence and absence of MPs for 20 min at 37 °C. Samples were washed, fixed with 2.5% glutaraldehyde and upon standard preparation procedure, visualized by SEM (n=3).
3.4.2.5 MP adhesion to collagen type I (bovine and human)

As measured by flow cytometry, MP adhesion to surface-bound bovine collagen type I (Fl.U.=83.1±15.7; n=4), was similar to that seen with human collagen type I (Fl.U.=83.8±11.2; n=4) (Figure 25a). MP adhesion to both bovine and human collagens was approximately 4 fold greater than that to albumin-coated surfaces, and this difference was statistically significantly different (p<0.01 by ANOVA) (Figure 25a). SEM images confirmed MP surface binding to both sources of collagen type I and suggested greater binding of MPs to the collagen-coated surfaces than had been indicated by flow cytometry (Figure 27, 31 and 32). Relative numbers of MPs visually quantitated on immobilized collagen SEM photomicrographs was 19-25 per 1 μm², that was 9-12 fold greater than that to albumin-coated surface (Table 3). Collagen-bound MPs appeared to be very small (less than 0.1 μm) compared to MPs bound to fibrinogen and fibrin, possibly accounting for this difference in flow cytometric results. Collagen-bound MPs (both bovine and human) were spherical and formed clusters of aggregates (in size 0.2-0.8 μm) (Figure 27, 31 and 32).

EDTA abolished MP adhesion to collagen type I CM (bovine at 95.0%±2.5; n=3) and human at 94.0%±3.1; (n=3)) confirming the metal-ion dependency of MP adhesion (Figure 25b; Table 2b). MP adhesion to surface immobilized collagen was also inhibited to the level of the EDTA control, by a monoclonal antibody against the platelet membrane collagen receptor GPIa/IIa (clone Gi9), at 92.0%±4.9 (bovine; n=3) and 92.5%±2.5 (human; n=4) inhibition, respectively [60-62,221]. These findings indicate that functional GPIa/IIa collagen receptors are present on the MP membrane surface. MP adhesion was collagen specific, since polyclonal affinity-purified anti-collagen type I antibodies inhibited MP adhesion to bovine (96.2%±1.6; n=4) and human collagen type I CM (96.0%±2.5; n=4) (Figure 25b; Table 2b).
The inhibition of MP adhesion and the loss of clusters of aggregated MPs on immobilized collagen type I in the presence of EDTA, monoclonal antibody against collagen receptor GPIa/IIa and anti-collagen type I polyclonal antibody to the level of the albumin control (0-3 MPs per 1 μm²), was confirmed by SEM studies (Figures 31 and 32).

In contrast to the albumin control, immunogold studies of MP/collagen-coated microspheres revealed an abundance of singular and clustered gold particles bound to biotinylated MPs, correlating with singular and aggregated MPs observed on both secondary images by reverse polarity SEM and common SEM images (Figure 29). Detection of gold particles on collagen-coated surfaces and their relation to secondary images confirms that the bound material on the collagen-coated surfaces originated from the biotinylated surface membrane of washed platelets and represented adherent platelet-derived microparticles.

These observations confirm our hypothesis that MPs adhere to collagen type I coated surfaces in a manner similar to that of platelets. MP binding to collagen type I (both bovine and human) was metal-ion dependent, involved the platelet collagen receptor GPIa/IIa and adhesion was collagen type I specific, similar to what was observed for platelets, in this assay system. Notably, MPs formed aggregates on collagen type I, as they did on immobilized fibrinogen and fibrin, which has not been previously reported. Surface-bound purified, biotinylated MPs were confirmed to be platelet-derived by streptavidin-gold identification of singular and clustered MPs on immobilized collagen type I (bovine or human).
Table 2b Inhibition studies of MP adhesion to immobilized collagen type I (bovine and human)

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</tr>
<tr>
<td>Polycl anticol ab</td>
<td>96.2±1.6</td>
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</table>

n=3-4 (mean±SEM); Monoclonal anti-GPIa/IIa antibody, clone Gi9 (GPIa/IIa mab Gi9); Polyclonal anti-collagen type I antibody (Polycl anticoll ab)

Results are expressed as % inhibition relative to MP adhesion to albumin-coated microsphere in the presence of EDTA (10 mM).
Figure 31 Scanning electron micrographs of MP adhesion to collagen type I (bovine) coated microspheres

Representative photomicrographs of collagen type I (bovine) coated microspheres incubated with EDTA (10 mM), monoclonal antibody anti-GPIa/IIa (anti-GPIa/IIa ab; 100 μg/ml), or polyclonal anti-collagen type I antibody (polycl anti-coll ab;125 μg/ml) in the presence and absence of MPs for 20 min at 37 °C. Samples were washed, fixed with 2.5% glutaraldehyde and upon standard preparation procedure, visualized by SEM (n=3).
Figure 32 Scanning electron micrographs of MP adhesion to collagen type I (human) coated microspheres

Representative photomicrographs of collagen type I (human) coated incubated with EDTA (10 mM), monoclonal antibody anti-GPIa/IIa (anti-GPIa/IIa ab; 100 µg/ml), or polyclonal anti-collagen type I antibody (polycl anti-coll ab; 125 µg/ml) in the presence and absence of MPs for 20 min at 37 °C. Samples were washed, fixed with 2.5% glutaraldehyde and upon standard preparation procedure, visualized by SEM (n=3).
3.4.2.6 Comparison of MP adhesion to protein-coated microspheres

As assessed by flow cytometry, MP adhesion was greater to fibrin-coated microspheres than to immobilized fibrinogen or collagen type I as assessed by ANOVA, followed by Tukey multiple comparisons post-test.

MP adhesion to fibrin (Fl.U.=180.3±13.7; n=4) was two fold greater than was the binding to immobilized fibrinogen (Fl.U.=94.1±9.1; n=4) or to collagen type I (bovine Fl.U.=83.1±15.7; n=4; or human Fl.U.=83.8±11.2; n=4) (p<0.001 by ANOVA; Figure 25a). However, there was no statistical difference between MP binding to either collagen (bovine and human) or fibrinogen-coated microspheres, as judged by flow cytometry (p>0.05 by ANOVA) (Figure 25a). Further, there was no statistical difference between MP adhesion to collagen type I bovine or human (p>0.05 by ANOVA) (Figure 25a).

MP adhesion to all surface immobilized ligands was cation-dependent and demonstrated similar levels of background fluorescence in the presence of EDTA (albumin Fl.U.=24.5±4.4; plain microspheres Fl.U.=26.6±5.7; fibrinogen Fl.U.=26.7±0.8; fibrin Fl.U.=29.8±0.5; collagen (bovine) Fl.U.=31.3±8.6; or collagen (human) Fl.U.=32.4±6.6; p>0.05 by ANOVA; n=4) (Figure 25b).

MP adhesion to fibrin-coated microspheres was 9-fold greater than that to albumin-coated microspheres control (9.2±2.5 X; n=4; p<0.001 by ANOVA). MP adhesion to fibrinogen (4.3±0.7 X; n=4; p<0.01), collagen type I bovine (3.9±0.8 X; n=4; p<0.01) and collagen type I human (4.1±0.9; n=4; p<0.01) were all about 4 fold higher than to the albumin control (Figure 10c). MPs binding to plain polystyrene surface microspheres wasn’t statistically different (1.3±0.2 X; n=4; p>0.05) from that to albumin control (Figure 33).

MP adhesion to the adhesive ligands fibrin, fibrinogen and collagen type I was supported by SEM studies that MPs bound most abundantly to the fibrin-coated microspheres (Figure
MP binding to collagen-coated surfaces was greater as assessed by SEM than had been suggested by flow cytometric analysis (Figure 27 and Figure 25a). The relative relationship of MPs visually quantified on different surfaces appeared to be: greater on fibrin, then collagen type I (both bovine and human), then fibrinogen and the least on plain polystyrene microspheres (Table 3).

MPs observed on protein-coated surfaces and on plain polystyrene beads were of very small size, ranging between 0.02-0.2 μm. Notably, MPs formed clusters or aggregates 0.2-08 μm in size on fibrin, fibrinogen and collagen type I-coated surfaces, but not on albumin or on the plain polystyrene surface. MPs bound to immobilized fibrin appeared to be "spread" or "flattened" when singular, or spherical when found in clusters of aggregates. MPs on fibrinogen and collagen surfaces appeared more spherical, both singularly and in aggregates. Collagen-bound MPs were less than 0.1 μm in size, smaller than those found on fibrinogen or fibrin-coated microspheres (Figure 27).

The inhibition of MP adhesion and the clustering on fibrinogen and fibrin-coated surfaces in the presence of EDTA, ReoPro, Integrilin and polyclonal anti-fibrinogen antibody was visually confirmed by SEM (Figures 28 and 30). The inhibition of MP adhesion and the absence of clusters of aggregated MPs on collagen type I coated surfaces (bovine and human) in the presence of EDTA, monoclonal antibody against GPIa/IIa and anti-collagen type I polyclonal antibody, was also confirmed by SEM (Figures 31 and 32).

Immunogold studies corroborated the flow-cytometric and SEM observations. Back-scattered images of MPs bound to fibrinogen, fibrin and collagen type I revealed abundant singular and clustered biotin-bound gold particles, corresponding to the singular and aggregated MPs observed on both secondary images by reverse polarity SEM and common SEM images (Figure 29). Immunogold studies confirmed that streptavidin-gold identified
particles seen on PCM/UCM originated from membrane surface of biotinylated washed platelets.

Table 3 Manual quantification of adherent MPs from SEM photomicrographs

<table>
<thead>
<tr>
<th>PCM/UPM</th>
<th>Flow cytometry</th>
<th>Rel. MP number/μm² (SEM: 20xK or 40xK)</th>
<th>MP size (μm)</th>
<th>Clusters of aggregates (size μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>1</td>
<td>2.3±0.4</td>
<td>2.7±0.4</td>
<td>0.05-0.1</td>
</tr>
<tr>
<td>Plain</td>
<td>1.3±0.2</td>
<td>6.8±0.4</td>
<td>8.3±0.4</td>
<td>0.05-0.2</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>4.3±0.7</td>
<td>18.0±1.2</td>
<td>23.0±0.6</td>
<td>0.05-0.1</td>
</tr>
<tr>
<td>Fibrin</td>
<td>9.2±2.5</td>
<td>28.0±1.2</td>
<td>37.3±2.2</td>
<td>0.05-0.2</td>
</tr>
<tr>
<td>Collagen I (bovine)</td>
<td>3.9±0.8</td>
<td>20.0±1.2</td>
<td>23.0±1.2</td>
<td>0.02-0.1</td>
</tr>
<tr>
<td>Collagen I (human)</td>
<td>4.1±0.9</td>
<td>18.7±0.9</td>
<td>25.0±1.3</td>
<td>0.02-0.1</td>
</tr>
</tbody>
</table>

n=3 (mean±SEM)

A comparison of the flow cytometric analyses (Fluorescence intensity relative to albumin) with the manual quantification of surface adherent MPs and the SEM MP morphology (MP size and presence of aggregates) are shown. MPs were counted manually from three blindly taken squares of 1 μm² from the SEM photomicrographs taken at enlargement of 20xK or 40xK. Results are presented as the mean±SEM (n=3).
Figure 33 MP adhesion to protein-coated microspheres relative to MP adhesion to albumin-coated microspheres as assessed by flow cytometry:

Plain, and albumin, fibrinogen, fibrin and collagen type I (bovine (1) and human (2)) coated microspheres were incubated with purified biotinylated MPs, labelled with secondary fluorochrome and evaluated by flow cytometry for evidence of MP adhesion. MP adhesion to protein-coated microspheres was quantified relative to the adhesion to albumin-coated microspheres (n=4±SEM; p<0.001*; p<0.01**; p>0.05*** see text pg. 150).
3.4.3 Contrasting MP and platelet surface adhesion

We have compared and contrasted the adhesion properties of purified MPs and of platelets, to the immobilized vascular ligands fibrinogen, fibrin and collagen type I. As demonstrated by flow cytometry, SEM and immunogold-SEM studies MPs, being rich in adhesive receptors such as GPIIb/IIIa and β1 integrin, were able to adhere, under static conditions, to immobilized fibrinogen, fibrin and collagen type I in a manner similar to that of platelets (Figures 21b, 25b, 28, 30, 31 and 32).

MP and platelet adhesion to immobilized fibrinogen, fibrin and collagen type I (bovine and human), similar to platelet adhesion, was metal-ion dependent. In addition, the mechanism of MP adhesion to PCM, involved the same platelet membrane surface receptors that are involved in platelet bindings. Both MP and platelet adhesion to fibrinogen and fibrin, involved β3 integrin, GPIIb/IIIa and was fully abolished by ReoPro and Integrilin indicating that MP GPIIb/IIIa receptor is functional. MPs, like platelets, adhered to collagen type I coated surfaces via the collagen receptor GPIa/IIa, since inhibition of binding was observed with the specific anti-GPIa/IIa monoclonal antibody (clone Gi9). Both MP and platelet adhesion to fibrinogen, fibrin and collagen type I was ligand specific, since affinity-purified polyclonal antibodies against either human fibrinogen or collagen type I, fully blocked MP adhesion to PCM.

Further, like platelets, MPs were observed by SEM imaging to form aggregates on immobilized fibrinogen, fibrin and collagen type I (bovine and human) surfaces, suggesting that MPs may express an additional function of membrane surface receptors. The ability of MPs to aggregate is a previously undescribed property of MPs that warrants further study.

Although MPs were able to adhere to the vascular ligands fibrinogen, fibrin and collagen type I in a manner similar to that of platelet adhesion, MP adhesion was qualitatively
different. It appeared that MP adhesion, relative to that of platelets, was greater to fibrin than to fibrinogen or collagen-coated microspheres (Figure 24, 27 and 33), while platelet adhesion was greater to immobilized fibrinogen. It is possible that MP GPIIb/IIIa, while in an active conformation, may have additional conformational changes that rendered it capable to bind, more efficiently, to immobilized fibrin than to fibrinogen.
3.5 Discussion

Platelet adhesion is a central event in supporting hemostasis and thrombosis at the site of vascular injury in the course of vascular wound repair. Upon platelet attachment to adhesive ligands (fibrinogen, fibrin, fibronectin, vitronectin, collagen, vWF) in the exposed subendothelial matrix, a series of simultaneous events, including platelet activation with membrane biochemical changes resulting in surface procoagulant activity, platelet aggregation, shedding of procoagulant membrane microparticles (MPs), culminate in thrombin and fibrin generation and thrombus stabilization [2]. In the course of these critical events, the role and the fate of platelets are well understood. However, the role and the fate of the platelet-derived MPs formed at this time are largely unknown, despite the fact that the MP surface resembles the activated platelet membrane in GPIIb/IIIa, GPIb, GPIIa and P-selectin expression. This "membrane duplication" may reflect the potential role of MPs in the circulation. A central question that has yet to be fully addressed is whether MP surface membrane receptors are functionally active and capable of interaction with surrounding cells and ligands. In this study, we have addressed, whether MPs can adhere to the vascular ligands fibrinogen/fibrin (GPIIb/IIIa) and collagen type I (GPIa/IIa), and have characterized the nature of this interaction.

This is the first study to fully characterize the adherence of human MPs to the physiologic vascular ligands fibrinogen, fibrin and collagen type I and to compare and contrast MP adhesion properties to those of human platelets. We show that MPs can adhere to fibrinogen, fibrin and collagen type I in a metal-ion dependent and, receptor and ligand-specific manner, similar to platelet adhesion. Furthermore, our study suggest that MPs are able to aggregate, a property to that of platelets. The anti-platelet GPIIb/IIIa agents, ReoPro and Integrilin, were efficient in abolishing both MP adhesion and MP aggregation to immobilized fibrinogen and
fibrin. This observation expands the role of these anti-platelet agents to include the inhibition of MP adhesion to immobilized fibrin and fibrinogen. MP adhesion to collagen type I was abolished by a monoclonal antibody against the α2-subunit of GPIα/IIa, establishing the presence and functionality of a collagen platelet receptor on the MP surface.

3.5.1 Platelet and MP adhesion to immobilized fibrinogen and fibrin

3.5.1.1 Platelet adhesion to fibrinogen and fibrin

Platelet adhesion to immobilized fibrinogen and fibrin has been studied extensively, mostly by two-dimensional flat surface assays or similar model systems and under conditions of shear stress [10,11,22,27,100]. We confirmed metal-ion and GPIIb/IIIa-dependent platelet adhesion to surface immobilized fibrinogen and fibrin under static conditions, using a unique, three dimensional adhesion assay involving protein-coated polystyrene microspheres (10 μm) and flow cytometry [55,100,199]. Further, we confirmed the flow cytometric analysis of platelet adhesion to microspheres by SEM studies that revealed layers of activated, spread platelets on fibrinogen and fibrin-coated microspheres. Since resting platelets were incubated with fibrinogen/fibrin-coated microspheres in the absence of any added agonist, our data indicate that platelets undergo contact activation in the presence of immobilized ligands. This observation is in agreement with the notion that the GPIIb/IIIa receptor does not need to be activated in order to interact with immobilized fibrinogen or fibrin [23,27]. Fibrinogen adsorption to surfaces induces conformational changes in the fibrinogen molecule that allow it to bind to unactivated platelets through the GPIIb/IIIa receptor, a phenomenon known as “substrate activation” [219,222]. These conformational changes of immobilized fibrinogen are detected by the same monoclonal antibodies that identify the exposed
neoepitopes (receptor induced binding sites RIBS I, II or III) that arise following fibrinogen binding to the activated GPIIIb/IIa receptor [219,220].

Our unique, flow cytometric, microsphere based, adhesion method proved to be a simple and efficient quantitative method for measuring both platelet and MP adhesion properties under static conditions. Compared to other adhesion assay methods that involve flat protein-coated surfaces, the advantages of this method are multifold. Advantages include: 1) increased contact surface to volume ratio for the interaction of platelets/MPs to immobilized proteins (125.6 cm²/cm³ compared to 10-25 cm²/cm³ available in 24 well plates or tubes, respectively); 2) the buoyancy of microspheres allows prolonged, three dimensional contact between platelets or MPs with the protein coat as compared to the two-dimensional flat surface system; 3) the larger platelet interactive surface permits shorter incubation times due to earlier platelet activation; 4) the preparation procedure is simple, and requires only small volumes of platelet/MP suspension (50-100 µl); 5) no radioactivity is involved; 6) post-incubational manipulation of samples (washing, elution steps) and thereby induced errors are eliminated; 7) relative fluorescence quantification (Fl. U.) of the surface of protein-coated microspheres by flow cytometry, as a measurement of platelet/MP adhesion, is a simple procedure whereby microspheres are first identified by size (FSC; 10 µm) and complexity (SSC), and then the fluorescent signal per bead of adherent cells is recorded; and 8) it provides an effective, quick and direct test to evaluate the inhibition of platelet/MP adhesion by a specific inhibitors (EDTA, ReoPro, Integrilin, anti-GPIa/IIa monoclonal antibody or polyclonal anti-fibrinogen and anti-collagen antibodies). However, this method does have limitations. Only the fluorescence signal of the non-adherent platelet membrane would be recorded, since some of the receptors would be involved in binding to the immobilized protein and would not be labelled by the detecting fluorochrome (antibody) or would be
covered with outside layers of platelets. Further, the potential for congregation of microspheres exists. Therefore any absolute platelet quantification might be potentially biased. However, relative platelet binding quantification is possible by gating single bead events by (FSC) as opposed to single cytometric events (Figure 18). The fluorescence signal associated with the surface of single beads then represents a measurement of the degree of platelets/MP adhesion to PCM/UPM. Therefore, the method remains simple, effective and the most sophisticated in assessing adhesion and inhibition of platelet/MPs to protein-coated surfaces.

3.5.1.2 MP adhesion to fibrinogen and fibrin. The role of MP membrane surface 

GPIIb/IIIa integrin

The GPIIb/IIIa receptor on the MP membrane has been reported to be in an inactivated conformation and unable to bind fibrinogen, as a result of its disconnection from the platelet cytoskeleton during the process of MP shedding [170]. It was suggested therefore that, MP GPIIb/IIIa is similar to that found on resting platelets, unactivated and unable to participate in adhesion to fibrinogen (or to other RGD ligands such as vitronectin, fibronectin or vWf) or to mediate particle-particle or platelet--MPs interaction [170]. In contrast to this observation, Sims et al., found that GPIIb/IIIa of MPs, generated upon platelet stimulation with thrombin plus collagen, was in an active conformational form and was able to bind PAC-1, an activation specific GPIIb/IIIa monoclonal antibody [21]. In addition, MPs were able to bind fibrinogen, as demonstrated by the RIBS monoclonal antibody (9F9), an antibody specific for membrane bound-fibrinogen, suggesting functional activity of the MP GPIIb/IIIa receptor [21]. These latter studies are in agreement with our observations that MPs are able to bind to both immobilized fibrinogen and fibrin, and that this adhesion is mediated through a
functional GPIIb/IIIa receptor, as adhesion was abolished by EDTA and by the anti GPIIb/IIIa blockers, ReoPro and Integriulin. We further proved ligand specificity by demonstrating that a polyclonal anti-fibrinogen antibody could completely block MP adhesion to both fibrinogen and fibrin.

Sporadic studies have tried to characterize the adhesion properties of MPs utilizing protein immobilization on flat surfaces and subsequent exposure to purified MPs. In one such study, MPs generated by different agonists (calcium-ionophore A23187, α-thrombin, SFLLRN, collagen or combination of thrombin and collagen) were exposed to ELISA-plates coated with fibrin, fibrinogen, collagen or vWF, demonstrated greater MP adhesion to fibrin and fibrinogen than to the other vascular ligands [136]. However, MP adhesion to immobilized fibrinogen/fibrin was only partially abolished (up to 45%) by the fibrinogen peptides RGDS and KQAGDV [136]. In two other studies employing the ELISA method, MP adhesion to fibrinogen-coated surfaces was abolished only about 50% by ReoPro [168]. In another study, adhesion of MPs to fibrinogen and fibronectin was abolished up to 90% by ReoPro indicating the GPIIb/IIIa dependency of adhesion [169]. Additionally, fibrin-adherent MPs have been observed by SEM during thrombus formation in vivo [136]. Our data confirm these observations.

The microsphere based, adhesion, flow cytometric assay that we used for platelet adhesion was applied to the characterization and comparison of MP adhesion to PCM/UPM. As discussed earlier, in contrast to experiments with flat protein-coated surfaces (ELISA) [136,168], in which surface area is limited, the microspheres provided for a much larger surface to volume ratio, making this assay more sensitive to adhesion. Further, since microspheres (10 μm) were of light density they remained buoyant for more than 15 minutes, allowing prolonged three dimensional contact between suspended MPs and immobilized
fibrinogen/fibrin without stirring. Flow cytometric analysis of these microspheres was easy, since they were many times larger than are MPs or platelets (10 μm). The increased fluorescence signal associated with surface adhesion of particles, compared to the fluorescence of albumin-coated microspheres allowed for the relative quantification of bound MPs on fibrinogen or fibrin-coated microspheres. Further, we were able to demonstrate complete inhibition of MP adhesion to fibrinogen and fibrin-coated microspheres by the addition of EDTA, ReoPro, Integrilin or of polyclonal anti-fibrinogen antibody by both flow cytometry and SEM imaging, in distinction to others [136,168] indicating receptor/ligand specificity for MP adhesion. MP adhesion to fibrinogen and fibrin was, as is platelet adhesion, metal-ion dependent and ligand specific. In addition, we demonstrated that MP adhesion to fibrinogen and fibrin occurred in the presence of a functional β3 integrin GPIIb/IIIa, in contrast to the study by Nomura et al [170] but consistent with other MP/adhesion studies [136,168,169].

Our SEM studies also indicated that MPs are able to aggregate under static conditions. To the best of our knowledge, this finding has not been previously observed. Clusters of aggregated MPs were observed on fibrinogen and fibrin-coated microspheres and were abolished by ReoPro and Integrilin, suggesting that MPs and platelets share similar agglutination properties that involve GPIIb/IIIa and fibrinogen. The aggregation of MPs in our system is likely mediated by fibrinogen either released from the fibrinogen-coated polystyrene surface (which we believe is highly unlikely since fibrinogen adsorbance to the surface has been described as very strong and irreversible) [219], or available upon platelet activation and MP generation by release from the internal α-granules fibrinogen pool. This latter hypothesis is supported by collagen MP adhesion experiments in which clusters of aggregated MPs were also observed by SEM, in the absence of immobilized fibrinogen.
Thus, the only source of fibrinogen could have been from α-granules, and released during platelet activation and MP generation. The release of the internal fibrinogen pool could also explain possible MP generation seen upon platelet-contact activation (Table 1c, Figure 22). MP-MP and potential platelet-MPs interaction require further study.

It has been previously suggested that fibrinogen is located on the membrane surface of MPs bound to GPIIb/IIIa receptor, even though GPIIb/IIIa receptor is disconnected from platelet membrane cytoskeleton [21,48]. If fibrinogen is already bound to membrane surface GPIIb/IIIa of MPs, then our data indicate that GPIIb/IIIa platelet receptors are not all saturated with fibrinogen (fibrin) upon washed platelet activation and MP shedding [76,86,184,185]. Alternatively, fibrinogen binding to GPIIb/IIIa on MPs, now disconnected from cytoskeleton, may be reversible, since we were able to completely eliminate GPIIb/IIIa mediated adhesion to immobilized fibrinogen/fibrin by the GPIIb/IIIa blockers ReoPro and Integrilin. For this to occur, either GPIIb/IIIa is free or unoccupied or ReoPro and Integrilin can compete off fibrinogen from GPIIb/IIIa and interfere with MP adhesion to immobilized fibrinogen/fibrin that requires free receptor [76,86,184,185].

Fibrin appeared to be a more adhesive surface for MPs than were other vascular ligands, that was in agreement with the one previous study that looked at this issue [136]. In contrast to MPs binding to fibrin, platelet adhesion was more prominent on fibrinogen than on fibrin in our system. We hypothesize that this increased binding of MPs to fibrin may result from unique conformational changes of the MP GPIIb/IIIa receptor that render it more avid for immobilized fibrin than fibrinogen, suggesting that platelet and MP GPIIb/IIIa may be subtly different. Intravascular growing thrombi consist of meshes of fibrin strands and aggregated platelets. It is possible that the preferential binding of MPs to fibrin strands at the site of growing thrombi may function to amplify locally the process of coagulation through
thrombin generation. The reduced ability of MPs to bind to fibrinogen and collagen type I at the site of vascular injury suggests additional potential ways to regulate the hemostatic processes.

3.5.2 Platelet and MP adhesion to collagen type I

3.5.2.1 Platelet adhesion to collagen type I

Collagen type I is the most ubiquitous fibre type in the human body and belongs to a group of highly platelet reactive collagens (together with types II, III, IV and VI) that have been studied for platelet reactivity in vitro under both static and flow conditions [25,70,102,103]. Collagen type I is a major constituent of the subendothelial matrix and is the first collagen to come in contact with platelets upon vascular injury [25,70,102,103]. Platelet adhesion to collagen type I has been studied previously using collagen-coated flat surfaces under different shear conditions. Platelet adhesion to collagen is complex, involving both Mg$^{2+}$-dependent (mediated by GPIIa/IIa) and independent (mediated by GPVI or GPIX platelet receptors) events that evolves in a “two-site, two-step” process involving distinct receptors and collagen recognition sites [25,60-62,70,72,103,223]. The helical structure of collagen is necessary for recognition by the platelet collagen receptors. Therefore, both polymeric (quaternary, fibrilar) and monomeric (tertiary, triple-helical) collagen type I support platelet adhesion [223-225]. Both platelet collagen receptors, integrin GPIIa/IIa and GPVI, have been shown to participate in “inside-out” platelet signalling upon initial contact with immobilized collagen [71-74]. However, the individual roles of these receptors, the contribution and the specific biochemical pathways in platelet activation must be still sorted out [18,25,62,73,226]. Platelet adhesion to collagen ex vivo varies considerably under different experimental conditions [25,70,71].
We have evaluated the adhesion of platelets to both bovine and human collagen type I (quaternary and tertiary collagen structure), in parallel with studies of platelet adhesion to fibrinogen and fibrin. The platelet medium contained physiological concentrations of Mg\textsuperscript{2+} and Ca\textsuperscript{2+} (1mM/2mM). As assessed by flow cytometry, platelets adhered to a similar extent to both bovine and human collagen type I. However, in both cases platelet adhesion to collagen was much less than that to immobilized fibrinogen and fibrin under identical experimental conditions, consistent with previous reports [10,22,23,100].

Further, platelet adhesion to both bovine and human collagen type I was inhibited (76-82%) by EDTA, indicating metal-ion dependency of adhesion and suggesting that up to 25% of platelet adhesion may be metal-ion independent. We suggest that other platelet collagen receptors, possibly including GPVI or GPIV as reported by others, may account for this binding [71,72,223]. GPVI has been demonstrated to recognize both the tertiary (triple-helical; monomeric) and quaternary (polymeric; fibrilar) structure of collagen in a metal-ion independent process and to be mainly responsible for platelet activation [25,69,72]. We have demonstrated contact platelet activation by immobilized collagen type I (bovine-quaternary and human-tertiary) by both flow cytometry and SEM. Nakamura et al., have shown that washed platelet adhesion to collagen-coated surfaces is a complex process that involves primary platelet adhesion through GPIa/IIa, followed by platelet activation through both the GPVI and GPIa/IIa receptors leading to conformational changes of GPIIb/IIIa receptor and platelet aggregation. These events could be blocked completely by the combination of anti-GPIa/IIa and anti-GPVI monoclonal antibodies, but not if just one monoclonal antibody was used [71]. Our observation is in agreement with these studies. We demonstrated collagen-induced contact platelet activation in the absence of any added agonist. Platelet activation events included up-regulation of GPIIb/IIIa, GPIb down-regulation, P-selectin expression and
MP generation (Table 1c). Further, surface-bound platelets were spread and activated as visualized by SEM (Figure 22).

Previous research indicated that platelet Mg²⁺-dependent adhesion to collagen is mediated predominantly through the integrin α2β1 (GPIa/IIa) and is inhibited with α2 subunit monoclonal antibody (clones 6F1 or Gi9) [61,62,103]. Our observation that up to 86% inhibition of platelet adhesion to collagen type I results from the addition of anti-GPIa/IIa antibody (Gi9) is consistent with these studies.

Thus, in our microsphere based adhesion assay and both by flow cytometry and by SEM, we have shown platelet adhesion to immobilized collagen type I that is ligand specific, metal-ion dependent and mediated by GPIa/IIa platelet membrane surface integrin.

3.5.2.2 MP adhesion to collagen type I. The role of the GPIa/IIa receptor

MPs adhered to collagen type I (bovine, human) in our assay system, in a manner similar to that of platelets. MP adhesion to collagen type I, is metal-ion dependent, since it was abolished by EDTA. MP adhesion to both bovine and human collagen type I was also GPIa/IIa specific, as it was eliminated with the monoclonal antibody against GPIa/IIa (Gi9), raised against α2 subunit. These findings substantiate the presence of the α2 subunit of the integrin GPIa/IIa on the membrane surface of MPs, which has not been previously demonstrated and indicate that the collagen receptor on MPs for collagen type I is integrin GPIa/IIa. The presence of β1 integrin (GPIIa) has been previously demonstrated on MP membrane and it is recognized to be part of the collagen (GPIa/IIa), laminin and fibronectin receptor [140]. However, GPIa (α2) subunit of designated collagen receptor has not been demonstrated to date on MP membrane surfaces. Other collagen platelet receptors (GPVI or GPIV) [25,61,62,69,227] have not yet been described on the surface membrane of MPs.
either. The potential role(s) of these platelet collagen receptors in MP formation remain to be determined.

Two previous studies have used an ELISA model to evaluate the adhesion properties of calcium-ionophore generated MPs to immobilized collagen type I coated flat surfaces [136,169]. Siljander et al. [136] demonstrated reduced MP adhesion to collagen type I compared to immobilized fibrin, consistent with our results. We have characterized for the first time however, that MP adhesion to collagen type I is metal-ion and integrin GPIa/IIa-dependent, and ligand specific, as assessed by both flow cytometry and SEM. Furthermore, SEM studies showed that MPs adherent to collagen type I had a distinct morphology: MPs were spherical, very small in size down to 0.02\(\mu\)m and rich in clusters or aggregates. As previously discussed the mediator of MP aggregation in the presence of collagen-coated microspheres may be endogenous fibrinogen released at the moment of MP generation. This notion requires further evaluation.

Thus, MPs are able to adhere to immobilized collagen type I under static conditions in a manner similar to platelets, via the membrane surface integrin GPIa/IIa and in a cation-dependent manner. Since the platelet interaction with collagen type I (as an abundant constituent of subendothelial matrix) is one of the first events in the normal hemostatic response, the ability of the MPs to bind to collagen type I might result in the local amplification of the coagulation process at the site of vascular injury, by direct binding to exposed collagen fibres.
3.5.3 Morphology of MPs by SEM

3.5.3.1 MP size

As visualized by SEM, MPs adherent to immobilized fibrinogen, fibrin and collagen type I coated surfaces were very small, ranging in diameter between 0.02-0.2 μm (0.1 μm on average). MPs were single entities or they formed clusters of aggregated MPs. When evaluated by flow-cytometry MPs have been reported to be smaller than 0.8 μm but singular particles smaller than 0.1 μm cannot be detected due to the limited resolution abilities of flow cytometers [107]. SEM and TEM studies of activated platelets generating MPs, reported the variable size of MPs (from 0.8 μm and smaller than 0.08 μm) [131,228]. However, an early SEM study reported average MP size of 0.1 μm that were generated from the tips of protruding pseudopodia upon collagen stimulation of platelets [132]. In addition, in previous studies in which platelets and MPs were separated, the average size of MPs was reported to be 0.1 μm, which is consistent with our observation [135,138]. We separated platelets and MPs by centrifugation and filtration techniques. We filtered MP through 0.8 μm filter, yet we did not observe particles larger than 0.2-0.3 μm in diameter by SEM studies of MP adherent to immobilized fibrinogen, fibrin and collagen type I. Whether larger MPs (0.8 μm) were generated at all or underwent secondary dispersion to a yet smaller particles once in contact with immobilized proteins is not known. It is conceivable, therefore that MP up to 0.8 μm in size recorded by flow cytometry, could be aggregates of smaller MPs. Alternatively, it is possible that larger particles were generated upon platelet stimulation, but were unable to adhere to immobilized fibrinogen, fibrin or collagen type I, and were therefore not observed by SEM.

There have also been attempts to correlate MP size and procoagulant activity [137,209]. Wenche et al. reported that in vitro generated MPs smaller than 0.1 μm were more pro-
coagulant, as measured by a phosphatidylserine dependent Russell Viper Venom Time (RVVT) [209]. Tans et al., however reported that MPs can be both procoagulant, due to the binding sites for prothrombinase complex assembly, or anticoagulant, depending on the presence of activated protein C on the membrane of MPs [125]. This raises the issue of whether the size of surface membrane generated MPs affects procoagulant activity.

Significant methodological differences exist in platelet and MP preparation that hamper the comparison of the physiological and biological properties of MPs among studies. MP preparation-separation techniques include filtration (0.8 μm filter) of activated platelets [135], differential centrifugation (10,000-16,000 xg for 1-10 min), differential centrifugation (2000 xg for 10 min) through a sucrose layer using supernatant rich in MPs [134,136] or prolonged ultracentrifugation (100,000-160,000 xg for up to 2 hours) whereby an invisible pellet was the source of purified MPs [138,190,229].

We separated MPs from platelets by a combination of centrifugation (8000 xg for 15 min) and filtration techniques (0.8 μm filter) and appeared to achieve an excellent separation of MPs from platelets, since no remnant platelets were observed in any of our SEMs. High purity of the MP populations was confirmed by flow cytometry and the rare events (less than 3%) that fell into the platelet gate most likely represented MP-MP aggregates, as seen by SEM. MP-MP interaction in suspension, may explain MPs larger than 0.2 μm seen by flow cytometry. The possibility of MP aggregates and coaggregation to platelets has been previously demonstrated in a flow cytometric study that involves native washed platelets and MP generated from biotinylated platelets [168]. This notion is consistent with the concept of a functional GPIIb/IIIa receptor on the MP membrane surface that is able to bind to immobilized fibrin(ogen), as demonstrated in this study and would explain the MP-MP or MP-platelet interactions observed by our SEM studies.
3.5.3.2 Morphology of MPs

This is the first study to characterize the morphology of MPs adherent to fibrin, fibrinogen and collagen type I-coated surfaces and to demonstrate that MPs appear to have distinct morphologies, depending on the ligand used.

MPs adherent to immobilized fibrinogen and collagen type I were spherical and small (down to 0.02 μm). MPs bound to collagen type I were numerous and very small (down to 0.02 μm) compared to fibrinogen and fibrin bound MPs, suggesting that they may have microparticulized further once in contact with immobilized collagen. Fibrinogen bound MPs were spherical and formed grape-like clusters of aggregates. MPs on fibrin consistently were more irregular in shape with tiny pseudopodia, and were “flattened” (size 0.1-0.2 μm on average) similar in appearance to spread platelets on the fibrin-coated microspheres in our studies.

Further, this is the first study to reveal large conglomerates of aggregated MPs. Aggregation is a distinct biological property of platelets [1,9]. We have now described that SFLLRN-induced MPs may have a similar ability. MP aggregates on immobilized fibrin, fibrinogen or collagen type I were between 0.2-0.8 μm in size and as discussed earlier, MP aggregation was probably mediated by fibrinogen released from the internal platelet pool at the moment of MP generation, a postulate that needs further evaluation.

3.5.3.3 The origin of MPs

Some researchers have reported two different size populations of MPs seen by SEM (one population between 0.08-0.2 μm in size and the other between 0.4-0.6 μm) [131,137]. Some have suggested that very small MPs (0.04-0.1 μm) may actually be derived from intracellular bodies and α-granules, while bigger microparticles originated from surface membrane
shedding [137]. In contrast, a recent study that employed atomic force and fluorescence microscopy to analyze thrombin and synthetic surface generated MPs found that MPs arose from platelet pseudopodia and had an average size of 0.125±0.021 μm [135]. Our flow cytometric, SEM and immunogold data indicate that MPs have originated from the platelet membrane surface. By biotinylating platelet membrane surface proteins, (presumably including GPIIb/IIIa, GPIb, GPIa/IIa, αvβ3), and secondary labelling with streptavidin-conjugated gold-particles, visualized by back scattered SEM, we have shown that MPs, in size of 0.02-0.2 μm are generated from the platelet membrane surface and not from internal platelet constituents. A similar platelet surface biotinylation procedure previously demonstrated the presence of platelet surface membrane receptors (GPIIb/IIIa and GPIb) on both platelets and MPs and documented, by crossed immunoelectrophoresis and parallel staining by enzyme conjugated avidin and Coomassie brilliant blue, that internal platelet proteins had not undergone biotinylation [146]. Further, the extensive work of Fox et al., regarding the platelet cytoskeleton adds to the notion that MPs are platelet membrane surface derived, since components of the platelet cytoskeleton (actin, actin binding protein, talin and myosin heavy chain) that cross connect through membrane GPIIb/IIIa receptor upon platelet activation, were extracted from MPs shed from platelet surface [34]. Our studies are consistent with these data and support the notion that MPs emanate from the platelet surface membrane.

3.5.4 Conclusions

In conclusion, SFLLRN-generated, purified, biotinylated MPs, were able to adhere in static conditions to immobilized fibrinogen, fibrin, collagen type I surfaces, in a manner similar to that by which platelets adhere to these vascular ligands. Further, we have shown
by a unique flow cytometric adhesion assay, SEM and immunogold SEM that MP adhesion to surface-bound fibrin, fibrinogen and collagen type I, similar to that of platelets, is metal-ion dependent, and receptor and ligand specific proving our postulate that platelets and MPs share similar adhesive properties to these vascular ligands.

MPs generated from the membrane surface of biotinylated washed platelets had on their surface functional platelet surface receptors that mediated MP adhesion to immobilized fibrinogen, fibrin and collagen type I in a cation-dependent manner.

The GPIIb/IIIa receptor mediated MP adhesion to immobilized fibrinogen and fibrin. MP adhesion and aggregation on immobilized fibrinogen and fibrin was ligand specific. The adhesion of MPs was abolished by the anti-platelet agents ReoPro and Integrilin, expanding the role of these agents to include inhibition of MP adhesion to fibrin/fibrinogen. In contrast to platelets, more MPs bound to immobilized fibrin than to fibrinogen.

MP adhesion to collagen type I was specific and mediated by the membrane surface integrin GPIa/IIa receptor. This is the first study to demonstrate both the presence and the function of α2 subunit of GPIa/IIa integrin as part of the MP membrane surface. MP adhesion to immobilized collagen type I was ligand specific.

Singular MPs were small and spherical on fibrinogen and collagen-coated surfaces (0.02-0.2 μm) and formed clusters or aggregates (0.2-0.8 μm). Spread, flattened, MPs were observed on the fibrin-coated surface. Our observations that MPs are able to aggregate and to bind to activated platelets are new and warrant further study.

Future studies are needed to assess MP adhesion under flow conditions and to evaluate other platelet binding ligands such as vWF, vitronectin or fibronectin.
Chapter 4  Summary and future directions

The clinical observation of circulating platelet-derived microparticles (MPs) in arterial and venous thrombotic disease or in clinical platelet activation states and their prothrombotic role in hemostasis and thrombosis, have made their study an important new area of vascular research. The goal of this study was twofold: 1) to elucidate the mechanism(s) of MP generation by the in vitro study of the role of the GPIIb/IIIa receptor and its binding ligands; and 2) to characterize the biologic adhesion properties of MPs and to compare them to those of platelets, under static conditions. Taken together, these studies will further elucidate the clinical physiopathological consequences of the presence of MPs and generate insights into ways how to prevent potential pathological prothrombotic events.

4.1  The role of fibrinogen in MP formation

4.1.1  Fibrinogen and GPIIb/IIIa in MP formation

We have postulated that fibrinogen, as the central GPIIb/IIIa binding ligand with its binding sites RGD and γ-chain H12, is essential in thrombin-induced platelet membrane microparticulization. Further, we postulated that, of all the vascular ligands that can bind to the platelet integrin GPIIb/IIIa with RGD specificity (fibronectin, vitronectin and vWF), it is fibrinogen in particular that is absolutely required for platelet microparticulation.

It has been previously demonstrated that the platelet β3 integrin receptor GPIIb/IIIa plays an essential role in the generation of procoagulant platelet microparticles and that platelets from patients with the hereditary bleeding diathesis, Glanzmann's thrombasthenia type I, (that lack the GPIIb/IIIa receptor), are markedly impaired in the generation of microparticles
This observation suggested that one or all of the GPIIb/IIIa (fibrinogen, vitronectin, fibronectin and vWF) binding, RGD-containing ligands, could be involved in platelet MP generation. In this study, we have characterized the central role of fibrinogen, its GPIIb/IIIa binding peptides (RGDS and γ-chain H12), the GPIIb/IIIa blocking agents (ReoPro and Integrilin) and of the GPIIb/IIIa monoclonal antibodies (P2 and SZ21) in MP generation by a flow cytometric method utilising platelet-rich plasma (PRP) and washed platelets (WP) stimulated with thrombin or PAR1 thrombin receptor activating peptide (SFLLRN).

4.1.2 Summary of conclusions

We have demonstrated that fibrinogen binding to the β3 platelet integrin receptor GPIIb/IIIa is absolutely required for thrombin and SFLLRN-induced human platelet microparticle formation. Both the α-chain RGD and the γ-chain H12 binding motifs of fibrinogen are required for microparticle generation. We speculate that the GPIIb part of the β3 integrin GPIIb/IIIa, that contains the binding site for fibrinogen γ-chain H12 with the cryptic AGD sequence is critical for platelet microparticulization since other RGD-containing ligands can occupy the GPIIb/IIIa receptor but do not support platelet MP generation. The relative roles of the fibrinogen γ-chain and α-chain peptides GPIIb/IIIa binding motifs in platelet MP formation requires further elucidation. Further, by comparing the relative effects of thrombin and of the SFLLRN in microparticle generation, we also demonstrated an additional thrombin effect in MP formation, possibly indicating a role for fibrin or for alternative thrombin-dependent signalling. We have demonstrated a new pharmacological effect of the anti-platelet agents (ReoPro and Integrilin) currently in clinical use, to include inhibition of MP generation. We hypothesize that this new function will result in the down regulation of procoagulant activity, by blocking the formation of the MP
catalytic surface that participates in thrombin generation. This expands the potential roles of these anti-aggregating agents to include a mechanism for anticoagulant activity.

4.1.3 The role of fibrinogen and GPIIb/IIIa in MP formation: Significance of these findings

With our observations we have expanded the role of the GPIIb/IIIa receptor and of its binding ligand fibrinogen, to include the generation of microparticles (MPs). MPs are known to be rich in binding sites for tenase (factors VIIIa and IXa of the coagulation cascade) and prothrombinase (factors Va and Xa) complex assembly and are considered as highly procoagulant in the circulation.

In our effort to elucidate the mechanisms of MP generation, we targeted the GPIIb/IIIa receptor with fibrinogen binding peptides (RGDS and γ-chain H12), monoclonal antibodies against GPIIIa (clone SZ21) and GPIIb (P2) and with the anti-aggregating pharmacological agents, ReoPro and Integrilin. All of these GPIIb/IIIa related agents abolished MP generation in a dose-dependent manner. The observation that γ-chain H12 is involved in MP generation has not been previously described. Work of others indicated that γ-chain H12, the GPIIb fibrinogen binding motif, is physiologically specific and more important in platelet adhesion and aggregation than is the RGD β3 fibrinogen binding motif [86,90-92]. Recent research indicated that each fibrinogen peptide moiety induced distinct functional consequences through “outside-in” GPIIb/IIIa signalling [43,177,201]. Further, that functional differences in “outside-in” signalling may exist among various ligands binding to GPIIb/IIIa through a common RGD specificity. However, many of the new GPIIb/IIIa blocking pharmaceutical agents were designed according to RGD-GPIIb/IIIa paradigm. There is concern in using RGD-based peptides or compounds as therapeutics, since there are
a large number of integrins that bind RGD-containing peptides (or RGD-containing ligands) carrying the potential for side effects due to this lack of specificity. Further, we demonstrated that the GPIIb/IIIa blockers, ReoPro and Integrilin do inhibit MP generation and suggest the premise that thus these drugs may have anti-coagulant effects in circulation. Potentially, the use of these drugs could be extended from their current use in interventional cardiology to conditions associated with platelet activation, thrombosis and elevated MP levels in the circulation or activated coagulation and fibrinolysis, such as heparin induced thrombocytopenia.

These findings give future directions for both basic and clinical research in the field and pharmaceutical industry.

4.2 Adhesive properties of MPs

4.2.1 Adhesive properties of MPs compared to platelets

We hypothesized that MPs share similar surface-bound ligand adhesive characteristics and functional counter receptors with platelets, since as far as we understand, MPs are derived from the platelet membrane. The adhesion characteristics of MPs in relation to platelets and the vascular ligands involved in MP adhesion are poorly characterized. We characterized and compared the adhesion of washed platelets and purified MPs to surface-bound proteins (albumin, fibrinogen, fibrin and collagen type I) using a unique static microsphere-based flow cytometric adhesion assay, scanning electron microscopy (SEM) and immunogold SEM.

We have shown that MPs and platelets share similar adhesive properties to the surface-bound ligands fibrinogen, fibrin and collagen type I under static adhesion conditions. Binding of MPs was greater to fibrin-coated surfaces than to fibrinogen and collagen. The
mechanisms of MP adhesion to surface-bound fibrin, fibrinogen and collagen type I, like that of platelets, involved GPIIb/IIIa and GPIa/IIa respectively, and was metal-ion dependent and ligand specific. The GPIIb/IIIa receptor was both present and functional on the surface of MPs and supported adhesion (and possibly aggregation) of MPs. GPIa/IIa was also, functional and present on the MP membrane surface and mediated MP adhesion to collagen type I. Singular MPs, as identified by SEM studies, were 0.02-0.2 µm in size, while aggregates of MPs on the surface of immobilized fibrinogen, fibrin and collagen type I of MPs were 0.2-0.8 µm in size. We demonstrated by biotinylation and immunogold SEM studies, the platelet membrane origin of generated MPs. Whether MPs adhere to immobilized vascular ligands under flow conditions and to other platelet adhesive ligands such as vWF, vitronectin and fibronectin will require further study.

4.2.2 MP adhesion biological properties: Significance of these findings

These studies are the first to more fully characterize the adhesive properties of MPs, albeit under static conditions. While MPs generated in the circulation might be different from SFLLRN-induced MPs in in vitro conditions, the characterization of their ability to adhere to the vascular ligands fibrin, fibrinogen and collagen type I, known to be both platelet adhesive and thrombogenic, is highly suggestive of potential clinical pathophysiological consequences of their existence, in vivo.

MPs may play a role in the amplification of the coagulation process at sites of vascular injury. Due to their ability to adhere locally to the mesh of fibrin, fibrinogen or exposed collagen fibrils at sites of vascular injury, MPs will amplify the process of coagulation through their high procoagulant surface-to-volume ratio, compared to intact platelets.
MPs further may disseminate procoagulant activity to sites distant from the place of their generation. Having low buoyant density, MPs are able to travel down-stream, disseminating procoagulant activity and amplifying coagulation through their ability to adhere to exposed collagen or deposited fibrinogen or fibrin at distant intravascular lesions (atherosclerotic plaque, injured blood vessel) thereby contributing to thrombin generation and activating the coagulation cascade.

The ability of MPs to aggregate (agglutinate) between themselves, revealed through our SEM studies, characterize MPs as sharing another behavioural property with platelets. We suggest that the coaggregation of MPs back to the surface of the platelet membrane may define MP-to-platelet signalling. These observations warrant future studies. Furthermore, in sporadic studies, MPs have been reported to interact with leukocytes and endothelial cells, suggesting potential signalling through these contacts as well [171,172]. Thus, MPs may have the role of modulating or regulating interaction between platelets, leukocytes and endothelial cells in the circulation upon platelet activation. This potential role of MPs requires future research.

Overall, the findings of this study may have a significant impact on the current view and concepts of the role of platelets and of platelet-derived MPs in the pathogenesis of hemostasis and thrombosis in vascular disease and in conditions characterized by platelet activation.

4.3 Future research directions

We hypothesized an essential requirement for fibrinogen and the GPIIb/IIIa receptor and confirmed their absolute role in thrombin (SFLLRN)-induced platelet microparticulization.
Further study is required to elucidate the relative roles of the fibrinogen peptide GPIIb/IIIa binding motifs (RGD and γ-chain H12) in platelet microparticulization. Their roles could be explored further by characterising the molecular mechanisms by which fibrinogen binding induces MP formation using mutant fibrinogen molecules defective in either sites or/and monoclonal antibodies to fibrinogen binding sites. The relative roles of other RGD GPIIb/IIIa binding ligands in MP generation could be explored further by using purified fibronectin, vitronectin or vWF, and washed platelets. Platelets from afibrinogenemic patients would also be useful in determining the relative functional role(s) of other RGD-containing ligands compared to fibrinogen. Alternatively, since afibrinogenemia is an extremely rare disorder, a "knock-out" (afibrinogenemic) mouse-model could provide valuable insights into these questions.

We hypothesized and proved that MPs share similar adhesive properties and functional adhesive receptors with platelets in respect to adhesion to fibrinogen, fibrin and collagen type I under static conditions. The adhesive properties of MPs to these ligands under flow conditions, as well as to the other vascular ligands (vWF, fibronectin, vitronectin, laminin or thrombospondin), remain to be elucidated. In addition, the ability of MPs to adhere to other platelet reactive collagens (types II, III, IV or VI) needs to be explored and the underlying mechanism(s) and potential differences to be characterized. A physiological in vitro flow model of endothelium and subendothelial matrix with controlled shear forces could be employed to further characterize MP adhesion properties. Our study also revealed that MPs have the ability to aggregate. This ability has not been previously described and requires further study. The flow cytometry, microsphere-based assay, together with SEM and immunogold SEM are currently the most sophisticated tools that could be used to elucidate the ability of MPs to aggregate between themselves and to the platelet surface membrane.
The nature of the MP-MP and MP-platelet binding could be further probed with blocking agents (fibrinogen peptides RGDS and γ-chain H12, GPIIb/IIIa blocking agents), monoclonal antibodies against specific epitopes to GPIIb/IIIa or fibrinogen) and with adhesive ligands (fibrinogen, vWF, vitronectin, fibronectin, laminin, thrombospondin), utilising native and biotinylated washed platelets. Finally, to further understand the role of MPs in the circulation it is important to define the interactions of MPs with other cells (endothelial cells or leukocytes) and the possible functional consequences of these interactions.

In summary, both mechanism(s) of MP generation and MP biological properties require further research with the final goal of better understanding the role(s) of MPs in the circulation and to develop strategies to control some of pathological consequences of MP generation.
References


22. Roth GJ: Platelets and blood vessels: the adhesion event. Immunology Today 13:100, 1992


41. Swords NA, Tracy PB, Mann KG: Intact platelet membranes, not platelet-derived microvesicles, support the procoagulant activity of adherent platelets. Arteriosclerosis and Thrombosis 13:1613, 1993


56. D'Souza S, Ginsberg M, Burke T, Plow E: The ligand binding site of the platelet integrin receptor GPIIb/IIIa is proximal to the second calcium binding domain of its α-subunit. J Biol Chem 265:3440, 1990


71. Nakamura T, Kambayashi J-I, Okuma M, Tandon NN: Activation of the GPIIb/IIIa complex induced by platelet adhesion to collagen is mediated by both alpha2beta1 integrin and GPVI. J Biol Chem 274, 1999


121. Smith BR, Rinder HM: Interactions of platelets and endothelial cells with erythrocytes and leukocytes in thrombotic thrombocytopenic purpura. Seminars Hemat 34:90, 1997


156. Gemmell CH, Ramirez MS, Yeo EL, Sefton MV: Platelet activation in whole blood by artificial surfaces: Identification of platelet derived microparticles and activated platelets binding to leukocytes as material induced activation events. J Lab Clin Med 125:276, 1995


203. Yeo EL, Misicijevic NM: Fibrinogen is an essential requirement for platelet MP formation. Blood 94:221a, 1999


the procoagulant response of single, collagen-adherent platelets. Thromb Haemost 81:782, 1999


