Platelet reactions in the non-self recognition of material surfaces

Marita Broberg

Göteborg 2001

Platelet reactions in the non-self recognition of material surfaces

Akademisk avhandling

som för avläggande av medicine doktorsexamen vid Göteborgs Universitet kommer att offentligen försvaras i hörsal Ivan Ivarsson, Anatomi och Cellbiologi, Medicinaregatan 3B, fredagen den 1 juni kl. 9.00

av

Marita Broberg

Fakultetsopponent: Professor Thomas Horbett, Bioengineering and Chemical Engineering, University of Washington, Seattle, USA

Avhandlingen baseras på följande delarbeten:

I. M Broberg, C Eriksson and H. Nygren
Early reactions between whole blood and hydrophilic or hydrophobic glass surfaces: effects of heparin and hirudin on von Willebrand factor binding and CD62P exposure on platelets
Colloids and Surfaces B:Biointerfaces 1998;11:123-130

II. H. Nygren and M. Broberg
Specific activation of platelets by surface-adsorbed plasma proteins
Journal of Biomaterial Science Polymer Edition 1998;9(8):817-831

III. M. Broberg and H. Nygren
Von Willebrand factor, a key protein in the exposure of CD62P on platelets
Accepted for publication in Biomaterials 2000

IV. M. Broberg and H. Nygren
GPIIb/IIIa is the receptor for platelet binding to hydrophilic and hydrophobic model surfaces
Manuscript submitted to *J Lab Clin Med*

V. M. Broberg and H. Nygren
Platelet response after single and consecutive exposure to plasma proteins
Manuscript

Platelet reactions in the non-self recognition of material surfaces

Marita Broberg
Department of Anatomy and Cell Biology
University of Göteborg, Box 420, SE 405 30, Sweden

Abstract

Every material introduced into the body in order to function in contact with a living tissue is defined as a biomaterial. Biomaterials are used throughout the world in an increasing rate to replace and augment organ functions. The insertion of an implant into the human body will initiate blood coagulation at the surface of the material.

After water and electrolytes have adhered to the surface a protein film starts to form. Platelets appear at the surface after a few seconds and the contact between platelets and the protein film is one of the initial steps in the non-self recognition of a foreign material.

The adhesion and reactions of platelets were investigated using both whole blood and isolated platelets. The platelets were isolated by a new method, where a reversible inhibition of platelet function was achieved by magnesium ions. The binding of platelets to the surface, their spreading, exposure of P-selectin, and release of granular contents were investigated.

The results show that the initial platelet adhesion to both hydrophilic and hydrophobic surfaces is mediated by the fibrinogen receptor GPIIb/IIIa. The binding of von Willebrand factor and exposure of P-selectin on the platelet surface are thrombin dependent events, but the spreading of platelets is not. Different protein coatings were seen to generate different responses from platelets. IgG was seen to fragmentize adherent platelets, leaving surface bound micro particles, while von Willebrand factor was seen to induce exposure of P-selectin on the platelet surface. The exposure of P-selectin on the platelet surface is of importance for the subsequent signalling between platelets and neutrophils, as neutrophils are known to bind to P-selectin. The release of granules, both α- and dense, was highest for platelets adhering to IgG and thrombin coated surfaces. Neither of these proteins was seen to have the same effects when added in soluble form to fibrinogen adherent platelets.

In conclusion, the initial platelet adhesion to surfaces in contact with blood is mediated by fibrinogen but the presence of other proteins in the film, as for example IgG and von Willebrand factor, generates very different platelet responses.

Key words: platelet, biomaterial, adhesion, granule, P-selectin, plasma protein, blood, activation, release.

ISBN 91-628-4763-5
Göteborg 2001

Department of anatomy and Cell Biology
University of Göteborg
Sweden

Platelet reactions in the non-self recognition of material surfaces

Marita Broberg

Göteborg 2001

Platelet reactions in the non-self recognition of material surfaces

Marita Broberg
Department of Anatomy and Cell Biology
University of Göteborg, Box 420, SE 405 30, Sweden

Abstract

Every material introduced into the body in order to function in contact with a living tissue is defined as a biomaterial. Biomaterials are used throughout the world in an increasing rate to replace and augment organ functions. The insertion of an implant into the human body will initiate blood coagulation at the surface of the material.

After water and electrolytes have adhered to the surface a protein film starts to form. Platelets appear at the surface after a few seconds and the contact between platelets and the protein film is one of the initial steps in the non-self recognition of a foreign material.

The adhesion and reactions of platelets were investigated using both whole blood and isolated platelets. The platelets were isolated by a new method, where a reversible inhibition of platelet function was achieved by magnesium ions. The binding of platelets to the surface, their spreading, exposure of P-selectin, and release of granular contents were investigated.

The results show that the initial platelet adhesion to both hydrophilic and hydrophobic surfaces is mediated by the fibrinogen receptor GPIIb/IIIa. The binding of von Willebrand factor and exposure of P-selectin on the platelet surface are thrombin dependent events, but the spreading of platelets is not. Different protein coatings were seen to generate different responses from platelets. IgG was seen to fragmentize adherent platelets, leaving surface bound micro particles, while von Willebrand factor was seen to induce exposure of P-selectin on the platelet surface. The exposure of P–selectin on the platelet surface is of importance for the subsequent signalling between platelets and neutrophils, as neutrophils are known to bind to P-selectin. The release of granules, both α- and dense, were highest for platelets adhering to IgG and thrombin coated surfaces. Neither of these proteins were seen to have the same effects when added in soluble form to fibrinogen adherent platelets.

In conclusion, the initial platelet adhesion to surfaces in contact with blood is mediated by fibrinogen but the presence of other proteins in the film, as for example IgG and von Willebrand factor, generates very different platelet responses.

Key words: platelet, biomaterial, adhesion, granule, P-selectin, plasma protein, blood, activation, release.

ISBN 91-628-4763-5
Göteborg 2001

Contents

Platelet reactions in the non-self recognition of material surfaces

Marita Broberg

Department of Anatomy and Cell Biology, University of Göteborg, Box 420,

SE 405 30 Göteborg, Sweden

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I - V):

I. M Broberg, C Eriksson and H. Nygren

Early reactions between whole blood and hydrophilic or hydrophobic glass surfaces: effects of heparin and hirudin on von Willebrand factor binding and CD62P exposure on platelets

Colloids and Surfaces B:Biointerfaces 1998;11:123-130

II. H. Nygren and M. Broberg

Specific activation of platelets by surface-adsorbed plasma proteins

Journal of Biomaterial Science Polymer Edition 1998;9(8):817-831

III. M. Broberg and H. Nygren

Von Willebrand factor, a key protein in the exposure of CD62P on platelets

Accepted for publication in Biomaterials 2000

IV. M. Broberg and H. Nygren

GPIIb/IIIa is the receptor for platelet binding to hydrophilic and hydrophobic model surfaces

Manuscript submitted to *J Lab Clin Med*

V. M. Broberg and H. Nygren

Platelet response after single and consecutive exposure to plasma proteins

Manuscript

Abbreviations

ADP	adenosin diphosphate
AMP	adenosin monophosphate
ATP	adenosin triphosphate
C	complement protein
CCD	charged couple device
CD	cluster designation
CTAP III	connective tissue-activating peptide III
D-PBS	Dulbecco´s phosphate buffered saline
FDA	fluorescein diacetate
FGF	fibrinogen growth factor
FITC	fluorescein isothiocyanate
GP	glycoprotein
HMWK	high molecular weight kininogen
HPTLC	high performance thin layer chromatography
LPA	lysophosphatidic acid
NMR	nuclear magnetic resonance
PDGF	platelet derived growth factor
PF4	platelet factor 4
PMN	polymorphonuclear
PRP	platelet rich plasma
PSGL-1	P-selectin glycoprotein ligand-1
RGD	peptide of Arg-Gly-Asp
vWF	von Willebrand factor
TBS	Tris buffered saline
TEM	transmission electron microscopy
TGF	transforming growth factor
TRITC	Tetra methyl rhodamine B isothiocyanate
VNR	vitronectin receptor

Introduction

In 1882 professor Bizzozero from Turin (Unknown, 1882) discovered a new cellular constituent of the mammalian blood, "Blutplattchen". Bizzozero described them as very pale, disc-shaped bodies, about one third the diameter of red blood cells and less numerous. Since then platelets have been studied for a hundred years and given an important role in both coagulation and hemostasis. More recently, platelet interactions with bacteria (Clawson and White, 1971; Joseph, et al, 1983), the role of platelets in migraine headache (Bic, et al, 1998) and Alzheimer disease (Chen, et al, 1995), and the involvement of platelets in inflammatory responses (Klinger, 1997; Mannaioni, et al, 1997; Page, 1989) have been studied. The interaction of platelets at biomaterial surfaces is an example of the latter.

Blood platelets

Blood is a fluid tissue consisting of both cells and plasma. A full-grown human has about 5 l of blood in a closed circulatory system. The cellular part of blood is divided into erythrocytes, leukocytes, and platelets. Leukocytes are further divided into PMN-cells (neutrophils, eosinophils, and basophils), monocytes, and lymphocytes (T and B). Platelets are the smallest cells in blood and the first to interact with blood interfaces. Each human possesses between 2 and 4 x 10^8 platelets/ml blood. Platelets are cytoplasmic fragments derived from megakaryocytes situated in the bone marrow. They lack nuclei and have a very reduced protein synthesis. The life span of platelets is about 10 days after which they are removed from the circulation by destruction in the spleen.

The discoid shape of platelets (2-4 μm in diameter) is maintained by a marginal bundle of microtubuli. The microtubuli are connected to an actin skeleton which coats the inner surface of the platelet membrane (Fox, 1993). Many membrane proteins are associated with this actin skeleton. A network of long actin filaments which bind myosin also exists throughout the cytoplasm of the platelet (Pollard, et al, 1977). Upon activation, rapid reorganisation of the cytoskeleton takes place, mediated by these two muscle proteins.

Platelets have two main types of granules, the α-granules and the dense granules. Upon release of the granular content, the granules are fused with the cell membrane in the

surface connected canalicular system. A very high concentration of the constituents are generated locally. Other structures in the platelets are lysosomes (sometimes referred to as λ-granules), peroxisomes, and grains of glycogen.

Blood coagulation

When a blood vessel is injured, a coagulation process immediately starts to minimise the blood loss. The coagulation could start and proceed via two different pathways, intrinsic and extrinsic, converging towards a common pathway (Macfarlane, 1964). The intrinsic pathway is initiated by contact with non-endothelial surfaces, mainly by adhesion of coagulation factor XII and HMWK to the surface. The extrinsic pathway begins with tromboplastin, released from damaged tissues, which activates coagulation factor VII in blood. Both pathways involve a chain of enzymatic reactions leading to conversion of soluble dormant plasma proteins into catalytically active enzymes, with the final step being formation of a fibrin clot (Jackson and Nemerson, 1980). Many of the reactions are dependent on calcium ions as cofactor. Both inhibiting and amplifying feedback systems are present in the coagulation system. At the formation of a clot, platelets are trapped in the fibrin network and activated by generated thrombin. This platelet trapping creates a physical barrier that limits blood loss, and the active platelets provide a surface functioning as cofactor for many of the steps in the coagulation cascade (Buller and Ten Cate, 1995). Activated platelets also release many of the coagulation proteins, generating a positive feedback.

Thrombin is regarded as the most central protein in the coagulation cascade (Dahlbäck, 2000) and thus inhibited by many anticoagulants. It is the main protein for positive feedback and is also important in the stabilisation of the clot (Narayanan, 1999).

Hemostasis

At the site of a vascular injury, blood coagulation and the formation of a platelet plug begin, as described above. This is the primary stage of hemostasis. Apart from being trapped in a fibrin network, platelets adhere to the vascular subendothelium exposed after tissue injury (Stel, et al, 1985). The adhesion generates a shape change and alteration of surface phospholipids, the latter mainly by exposure of phosphatidyl serine (Bevers, et al, 1982). A procoagulant surface is exposed, permitting binding of

coagulation factor X to coagulation factor Va and a complex is formed that accelerates the thrombin generation 1000 fold locally (Sims, et al, 1989).

The second stage of hemostasis is platelet aggregation. Aggregation is mainly achieved by fibrin and fibrinogen binding to GPIIb/IIIa, forming bridges between platelets (Ruggeri, 1994). Release of ADP, serotonin, and calcium from activated platelets further stimulates the platelets to aggregate (Andrews and Berndt, 1998). At this time, binding and activation of complement is also seen on the platelet surfaces (Devine, 1992). When platelets release their α-granular contents, coagulation factors become bound to the platelet surface, ensuring the formation of fibrin locally to stabilise the hemostatic plug (Sims, et al, 1989). Thrombospondin, vWF, and fibrinogen are also exposed bound to the platelet surface, enabling the formation of bridges between the platelets (Ruggeri, 1994). This network formed also traps erythrocytes. Coagulation factor XIII is also released from the platelets, stabilising the clot by fibrin cross-binding. A final contraction of the actin and myosin in platelets retracts the clot (Pollard, et al, 1977). Platelets and released granular contents contribute to the recruitment and activation of leukocytes to the site of injury.

Platelets in inflammation

During an inflammation in the human body, the incidents of thrombosis are increased because of enhancement of the hemostatic system and of platelet functions. However, the inflammation does not only affect the platelet reactivity, the platelets also take an active part in the process of inflammation and the repair of tissue that follows. Apart from accelerating coagulation and fibrinolysis, platelets release adhesive proteins, produce chemotactic substances, and release factors that alter the vascular permeability. Some of the chemotactic substances improve binding of leukocytes to the vascular endothelium (Klinger, 1997) and the increased vascular permeability facilitates the diapedesis of leukocytes (Mannaioni, et al, 1997), leading to neutrophil accumulation at the site of injury.

Among smokers, platelet activation is pronounced with elevated levels of serotonin receptors (Markovitz, et al, 1999), and clinical studies have shown that platelet activation is a feature of allergic asthma (Coyle, et al, 1990; Knauer, et al, 1981; Page, 1989). Both indicate a role of systemic interactions between platelets and inflammatory responses in the human body.

Platelet interactions with immunoglobulins

Platelets have a number of systems and receptors for uptake, release, and metabolism of vasoactive substances (Kieffer and Phillips, 1990). Immunoglobulins for example, are endocytosed from the plasma by platelets and stored in the α-granules (Harrison and Cramer, 1993). Platelets have two types of Fc-receptors on their surface, for IgG (Rosenfeld, et al, 1985) and for IgE (Page, 1989), indicating a role for platelets both in the humoral immune response and in allergy reactions. Opsonised microorganisms and mixed aggregates with IgG have been shown to initiate platelet aggregation and stimulate granular release (Clawson and White, 1971; Klinger, 1997).

In lower organisms, platelets have a much bigger immunological role by aggregation around foreign invaders and releasing cytotoxic substances to remove pathogens from the circulation (Page, 1989). Rat platelets have been shown to kill the parasite *Schistsoma mansoni* by an IgE-dependent mechanism (Joseph, et al, 1983). Activation of the IgE receptor on platelets results in a production of cytotoxic free radicals (Page, 1989).

Platelets and complement interactions

Platelets also interact with the complement system. They can modulate the action of complement through membrane proteins like all endogenous cells, but also physically respond to complement activation, from C1q binding to membrane attack complex regulation (Devine, 1992).

The C1q receptor on the platelet surface was initially identified by Peerschke and Ghebrehiwet (1987). Unstimulated platelets have approximately 4000 copies of this receptor on the surface. The platelet receptor for C1q has been shown to bind in a magnesium dependent manner. The receptor has also been shown to bind to collagen, due to structural similarities (Peerschke and Ghebrehiwet, 1990). Apart from intervening in the platelet response to collagen, aggregated C1q has been shown to stimulate platelet aggregation and release reactions (Cazenave, et al, 1976). This effect might be mediated by the co-operative interaction between the platelet IgG receptor and the C1q receptor (Peerschke and Ghebrehiwet, 1997).

Platelets have also been shown to have a distinct receptor for C3dg, i.e. complement receptor 4, indicating that platelets are capable of binding the final proteolytic cleavage

fragment of C3 that is covalently attached to the target after complement activation (Vik and Fearon, 1987).

Both decay-accelerating factor, controlling the formation and stability of C3 convertase, and C8 binding protein have been found in the platelet cell membrane. These two proteins protect the platelets from lysis by the complement system (Blaas, et al, 1988).

Histamine

Histamine is one of the proteins stored in platelet dense granules (Brown, et al, 1980; Klinger, 1997) It is probably the most ancient mediator of inflammation. It has effects on a variety of cells, i.e. platelets, leukocytes, endothelial cells, and vascular smooth muscle cells, generating a systemic inflammatory response. Histamine also exerts an anti-inflammatory effect due to generation of nitride oxide by endothelial cells (Mannaioni, et al, 1997).

Platelet adhesion receptors and integrins

Platelets have a number of different surface receptors (Kieffer and Phillips, 1990), some of which are presented in Table I. Many of these receptors are used for immuno-detection named by their CD name.

Table I

Platelet receptors	Other names	Ligands
FcγII-recptor	CD32	IgG
GPIV	GPIII, CD36	collagen, thrombospondin
integrin $\alpha_2\beta_1$	GPIa/IIa	collagen, laminin
integrin $\alpha_{IIb}\beta_3$	GPIIb/IIIa, CD41/CD61	fibrinogen, RGD-sequence
integrin $\alpha_5\beta_1$	GPIc/IIa	fibronectin
integrin $\alpha_v\beta_3$	VNR, CD51/CD61	vitronectin, RGD-sequence
integrin $\alpha_6\beta_1$	GPIc´/IIa	laminin
P-selectin	CD62P, GMP140, PADGEM	PSGL-1
PECAM-1	GPIIa, CD31	endothelium
thrombin receptor	GPIb/V/IX, CD42	thrombin, vWF

There are two well known inherited bleeding disorders connected to platelet receptors. The first is Glanzmann´s thrombasthenia, missing GPIIb/IIIa, and the second is Bernard Soulier syndrome, where GPIb/V/IX is non functional (reviewed by George et al, 1984).

The fibrinogen receptor

The most common receptor is the fibrinogen receptor with 50 000 copies per platelet (McEver, et al, 1983). It is an integrin consisting of an α- and a β-chain ($\alpha_{IIb}\beta_3$) and most commonly referred to as GPIIb/IIIa. GPIIb/IIIa is a calcium dependent complex (Fujimura and Phillips, 1983; Kunicki, et al, 1981) and less than micromolar concentrations of Ca^{2+} lead to dissociation of the complex with lost functionality (Jennings and Phillips, 1982; Fitzgerald and Phillips, 1985).

GPIIb/IIIa has two different binding sites for fibrinogen, the RGD-peptide binding site located in the β-integrin, amino acid 109-171 (D'Souza, et al, 1988), and a site located in the α-integrin which is able to bind the γ-chain of fibrinogen (D'Souza, et al, 1990). There are also two functionally different states of GPIIb/IIIa (Savage and Ruggeri, 1991), which can be detected by the monoclonal antibody PAC1 (Shattil, et al, 1985). The "resting" form of GPIIb/IIIa is able to bind to immobilised fibrinogen (Coller, 1980) via the α-integrin (Savage and Ruggeri, 1991). On activated platelets GPIIb/IIIa binds the RGD-peptide in various plasma proteins, both soluble and surface bound. The activation of GPIIb/IIIa could be achieved by soluble mediators as thrombin (Minckes, et al, 1994) and ADP (Savage and Ruggeri, 1991), or by ligand binding to other protein receptors, e.g. vWF binding to GPIb/V/IX (Savage, et al, 1992; Wu, et al, 1996). The pathway of receptor activation remains to be defined, but a conformational switch mechanism for the cytoplasmatic tail of the α-integrin has been detected by using NMR-studies (Vinogradova, et al, 2000), and there is another study suggesting that this change is calcium dependent (Andrews, et al, 1997).

Receptors topographically associated to GPIIb/IIIa

Topographically associated to GPIIb/IIIa is CD9 (Brisson, et al, 1997; Slupsky, et al, 1997) and the FcγII-receptor (Berndt, et al, 1993). The real purpose of CD9 is still unknown but antibodies binding to CD9 are known to activate platelets through complex binding to the FcγII-receptor (Kuroda, et al, 1995).

The FcγII-receptor is a low affinity receptor of 40 kDa (Rosenfeld, et al, 1985) which is phosphorylated upon ligand binding (Chacko, et al, 1994). Platelets have also been

shown to release a complete and a C-terminal truncated form of the FcγII-receptor at the time of α-granular release (Gachet, et al, 1995).

Thrombin receptors

Another important receptor on the platelet surface is the thrombin receptor, GPIb/V/IX. There are actually two thrombin receptors, GPIb/V/IX and a seven transmembrane domain receptor, working in synergy for optimal activation of platelets (Greco, et al, 1996). GPIb/V/IX is a leucine rich glycoprotein and the only platelet receptor known to be directly associated with actin binding proteins in the membrane skeleton (Andrews and Fox, 1991). After thrombin binding, the GPV part of the receptor is hydrolysed and a water soluble fragment is released (Berndt and Phillips, 1981). Under high physiological stress, the main function of GPIb/V/IX is to be the initial receptor for platelet adhesion to immobilised vWF (Ikeda, et al, 1991; Savage, et al, 1992), exposed in the subendothelium after vascular rupture (Stel, et al, 1985). Binding of vWF to GPIb/V/IX triggers intracellular signalling pathways, calcium flux, and activation of GPIIb/IIIa (Clemetson, 1995; Ikeda, et al, 1991).

Intracellular effects of ligand binding to platelet receptors

Many of the platelet receptors are linked to G-proteins. G-proteins are guanine nucleotide-binding regulatory proteins and modulate the interactions between receptors at the cell surface and effector molecules in the cell. The effector molecules are generally enzymes which generate second messengers in the cell. The most important G-protein regulated enzymes in platelets are adenelyl cyclase, phospholipase C, and phospho-lipase A_2 (Marcus, 1999). The activation of these enzymes starts phosphorylation cascades in the cells. Phosphorylation and dephosphorylation systems are very important for the functionality of many platelet receptors. For example, upon ligand binding, the FcγII-receptor is phosphorylated leading to a physical association with the protein kinases $p72^{syk}$ (Chacko, et al, 1994) and $pp125^{FAK}$ (Haimovich, et al, 1996b). This association also leads to phosphorylation of these proteins (Haimovich, et al, 1996b). Phosphorylation of $pp125^{FAK}$ via protein kinase C has also been detected after fibrinogen binding to GPIIb/IIIa (Haimovich, et al, 1993).

Platelet granular release

In response to various physiological stimuli, such as thrombin, ADP, collagen, epinephrine, and serotonin, platelets undergo a shape change, from discoid to spherical, and produce filipodial extensions. After centralisation of the granules by actin – myosin interactions (Fox, 1993), a release reaction takes place. Platelet aggregation is not necessary for release to occur in response to strong stimuli (Triplett, 2000).

α-granules

The α-granules function as repository for proteins that were synthesised in the megakaryocyte, e.g. PF4, β-thromboglobulin, and vWF, and also for proteins endocytosed by the platelet or megakaryocyte from plasma, e.g. fibrinogen, albumin, and immunoglobulins (Harrison and Cramer, 1993). Some other proteins stored in α-granules and released upon platelet activation are fibronectin, vitronectin, thrombospondin, CTAP III, PDGF, TGF-α and β, basic FGF, complement proteins D and H (Devine, 1992), histidine-rich glycoprotein, plasminogen, and coagulation factors V and VIII (McNicol and Israels, 1999).

P-selectin is present in the membrane of platelet α-granules and in the Weibel-Palade bodies of endothelial cells. This selectin is exposed on the platelet surface after activation through α-granular release (Hsu-Lin, et al, 1984; McEver and Martin, 1984; Stenberg, et al, 1985). The main function of P-selectin is to mediate adhesion of neutrophils and monocytes to activated platelets and endothelium by a Ca^{2+}-dependent mechanism (Larsen, et al, 1989). Circulating degranulated platelets lose exposed P-selectin rapidly but are still otherwise functional (Michelson, et al, 1996). P-selectin has also been reported to be present in the membrane of dense granules (Israels, et al, 1992).

The membrane of α-granules also contain GPIIb/IIIa (Cramer, et al, 1990; Wencel-Drake, et al, 1986), which is redistributed to the platelet surface at the time of activation, increasing the pool of fibrinogen receptors and thus promoting aggregation.

Dense granules

Platelets contain three to eight dense granules (McNicol and Israels, 1999). The release of dense granules generally requires more stimulation of the platelets than the release of α-granules. Thrombin, thromboxane analoges, ADP, and collagen cause dense granular

release (McNicol and Israels, 1999). The exocytosis of the granules requires ATP, both as cofactor for protein phosphorylation and by priming the granules prior to release (Morimoto and Ogihara, 1996). The dense granules contain serotonin (5-hydroxy-tryptamine), ADP, ATP, calcium, and pyrophosphate, all inducers of platelet activation (Holmsen and Weiss, 1979).

Serotonin is absorbed from the blood plasma by platelets and released unchanged during granular release (Holmsen and Weiss, 1979). It is a relatively stable protein in plasma and increased blood levels of serotonin and platelet activation are seen among patients with Alzheimer disease (Sevush, et al, 1998). Serotonin is also a vasoconstrictive substance and reduces flow at the site of an injury, thereby limiting blood loss (McNicol and Israels, 1999).

The ratio of ATP to ADP in dense granules are 2 to 3 and are metabolically inert for the platelets (Holmsen and Weiss, 1979). After secretion, both ATP and ADP is converted to AMP by neutrophils. AMP, in contrast to ATP and ADP, has an inhibitory effect on neutrophils (Aziz, et al, 1997). ADP is important in the aggregation response of platelets to antagonists as well as in the activation of neutrophils (Bazzoni, et al, 1992).

Calcium secreted by platelets contributes very little to the plasma concentration but the release may be of importance in tightly packed platelet aggregates. Calcium in the dense granules is known to form tight complexes with ADP, ATP, and pyrophosphate, allowing storage of higher concentrations of the ion (Holmsen and Weiss, 1979).

In the same way as α-granular membranes contain GPIIb/IIIa, dense granules have been shown to contain receptors for adhesive proteins which are exposed on the platelet surface after exocytosis. This leads to an expansion of the surface pool of adhesive receptors on activated platelets (McNicol and Israels, 1999).

Lysosomes, or λ-granules

Lysosomes are common cell organelles with a function in cell digestive systems and autolytic processes (Polasek, 1989). There are different forms of lysosomes and platelets contain primary lysosomes that can be secreted. This secretion is only observed after strong stimuli by high concentrations of thrombin or collagen (Metzelaar and Clevers, 1992). The lysosomal granules contain acid phosphatase, proteolytic enzymes, β-glucuronidase, and cationic proteins (Clawson and White, 1971; McNicol and Israels, 1999), all which have a potentially anti microbial effect. These enzymes are present in a latent form and their full activity is only achieved after disruption of the lysosomal

membrane (Polasek, 1989). In the same way as α- and dense granules, lysosomes contain lysosome associated membrane proteins that are exposed on the platelet surface after release of the granular contents (Metzelaar and Clevers, 1992).

Other platelet responses to stimuli

Apart from granular release, platelets show a number of other responses to stimuli. For example, formation of thromboxane and prostaglandins is a normal response to collagen stimuli (Marcus, 1999).

Another platelet response is the generation of lipid mediators. Lysophosphatidic acid, the smallest and simplest phospholipid, is released by platelets and evokes a wide range of biological effects (Moolenaar, 1994). A number of different forms of LPA have been identified after thrombin stimulation of platelets (Gerrard and Robinson, 1989) and after blood clotting (Eichholtz, et al, 1993). LPA causes smooth muscle contraction, platelet aggregation, fibroblast proliferation, etc., and may participate in the natural wound healing process (Eichholtz, et al, 1993). LPA is also known to accumulate in atherosclerotic plaques (Siess, et al, 1999), suggesting an involvement of platelets in atherosclerosis.

Many stimuli also generate an increased procoagulant activity of the platelets. This is a result of exposure of aminophospholipids, especially phosphatidylserine, on the platelet surface (Bevers, et al, 1982; Thiagarajan and Tait, 1991). Coagulation factors Va and Xa are assembled with phosphatidylserine into the prothrombinase complex on the platelet surface (Sims, et al, 1989), accelerating thrombin generation.

Strong agonists also generate release of microparticles from platelets. Formation of terminal swellings of platelet pseudopod, finally generating exocytosis, is believed to be the origin of these particles (Crawford, 1971). The procoagulant activity is enriched in the microparticles and most of the procoagulant surface generated by platelet activation is thus lost. During thrombosis, microparticles associate with fibrin, forming a procoagulant and negatively charged network (Siljander, et al, 1996). The protease calpain, known to give rise to LPA, has also been detected in active form on microparticles (Pasquet, et al, 1996).

Cross-talk between platelets and leukocytes

One of the more important roles for platelets during inflammation is to modulate leukocyte functions by cross-talk. Platelets have been found to markedly affect neutrophil behaviour as far apart as adhesion (Iwabuchi, et al, 1998) and phagocytosis (Zalavary, et al, 1996). Many peptide and lipid factors released from platelets are chemotactic for leukocytes. PF4, for example, is chemotactic to neutrophils (Aziz, et al, 1997) and monocytes (Osterman, et al, 1982), while LPA is only chemotactic to monocytes (Zhou, et al, 1995).

An example of close co-operation between platelets and neutrophils is CTAP III, released from platelet α-granules upon activation. CTAP III is a cleavage product of platelet basic protein and to some degree cleaved in the platelets, forming β-thrombo-globulin before release (Niewiarowski, et al, 1980; Varma, et al, 1982). Both β-thrombo-globulin and CTAP III are further cleaved (Castor, et al, 1989) by neutrophil released cathepsin G (Brandt, et al, 1991; Cohen, et al, 1992) to form neutrophil activating peptide-2, a very potent neutrophil activator (Walz, et al, 1991). Cathepsin G is also a platelet activator, generating an increase in the release of α-granules (Evangelista, et al, 1991).

This latter interaction is an example of neutrophils affecting platelets. Another example of this is the release of nitride oxide by neutrophils (Salvemini, et al, 1989). Nitride oxide is a platelet inhibitor which prevents platelet activation. Today, NO-releasing polymers are used in vascular grafts to increase biocompatibility (Pulfer, et al, 1997).

Many interactions between platelets and neutrophils are mediated by close contact through adhesion receptors and proteins. For example, fibrinogen bound to platelets are known to interact with CD11c/CD18 on neutrophils, inducing an oxidative burst (Ruf and Patscheke, 1995).

P-selectin is the only selectin present on platelets (about 10 000 copies/platelet) and only after granular release, as described earlier. It interacts with PSGL-1 on neutrophils (Sako, et al, 1993) and monocytes (Furie and Furie, 1995) rendering close contact between platelets and these cells. P-selectin also mediates leukocyte rolling on activated platelets and endothelium after vascular injury (McEver and Cummings, 1997). Binding of P-selectin to PSGL-1 induces activation of CD11b/CD18 (Evangelista, et al, 1999). A soluble form of P-selectin exists, which might be of importance in the prevention of

systemic inadvertent activation of neutrophils. The m-RNA for this form of P-selectin has been found both in platelets and endothelial cells (Dunlop, et al, 1992).

Biomaterials

The definition of a biomaterial is very vague. Any material (natural or man made) that is introduced into the human body to perform a certain function can be regarded as a biomaterial. The most important properties of the biomaterial vary between different locations in the body. However, wherever into the body this material is introduced a bleeding will initially be rendered. On most places a short inflammatory response before acceptance would be desirable. On other places, for example in the use of vascular grafts to replace blood vessels, a reaction as small as possible on the luminal side is preferable. As a result of this, very different qualities of the materials are desired, and to fulfil this there are a variety of different types of biomaterials.

When a biomaterial is introduced into coagulating blood there is a rapid adsorption, change, and replacement of proteins at the surface (Vroman and Adams, 1969). On hydrophobic biomaterials, fibrinogen is accumulated after blood exposure (Nygren, et al, 1988), while on hydrophilic surfaces the initial fibrinogen adsorption is replaced by factor XII and HMWK (Vroman, et al, 1980), leading to thrombin generation. Hydrophilic surfaces are also known to activate complement (Elwing, et al, 1986). This might be via IgG interactions, as IgG is known to activate complement on surfaces (Tengvall, et al, 1996). An indication for this is that neutrophil adhesion to hydrophilic surfaces has been shown to be mediated by the FcγIII-receptor (Eriksson and Nygren, 2001a).

Platelets adhesion to biomaterial surfaces

After five seconds of blood – biomaterial contact, platelets can be detected on the surface. Neutrophils begin to appear at the surface after about eight minutes (Nygren, et al, 1997), but at this point an intricate series of events has already started.

Many plasma proteins immobilised on different surfaces function as adhesion molecules for platelets by binding to the surface receptors discussed earlier. Different protein coatings have been shown to generate very different responses. The initial adhesion to surfaces under static conditions has long been believed to be mediated by fibrinogen (Zucker and Vroman, 1969; Stanford, et al, 1983). A reason for this is that the initial

adsorption events are dominated by fibrinogen (Baier and Dutton, 1969; Vroman and Adams, 1969). Numerous attempts have thereafter been made to minimise fibrinogen adsorption to biomaterial surfaces. More recently, an increased interest in the activation of platelets has emerged, and simply measuring the amount of adhered platelets to a surface is not regarded as a good measurement of blood – biomaterial compatibility anymore.

Platelet reactions to immobilised proteins

Early studies of single protein coatings revealed an extensive release of platelet constituents and aggregation after platelet exposure to an IgG coated surface (Packham, et al, 1969). More recently, adhesion of platelets to surfaces coated with IgG have been shown to generate very special morphologies, leading to formation of surface bound microparticles and fragmentation of the platelets (Broberg and Nygren, 1998). The platelets show high formation of filipodial extensions and a loss of organisation in the intracellular structure (Figure 1).

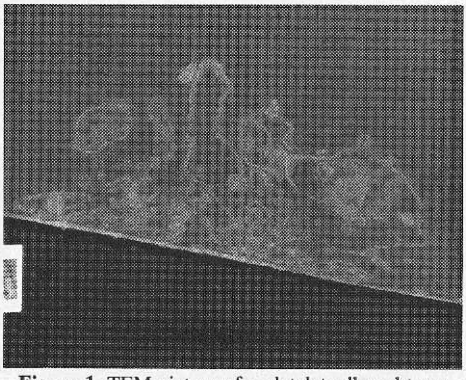

Figure 1. TEM picture of a platelet adhered to an IgG coated hydrophobic glass surface. Magnification x 17 000.

Collagen is one of the proteins known to mediate platelet adhesion after vascular rupture (Diaz-Ricart, et al, 1996), and platelet morphologies similar to those seen on surfaces coated by IgG have been detected on surfaces coated with collagen (Heemskerk, et al, 1997). Platelet interactions with collagen can however be prevented by C1q (Cazenave, et al, 1976), a more interesting protein from a biomaterial aspect, as C1q is a plasma protein. Platelet interactions with surface immobilised C1q has been shown to result in activation of GPIIb/IIIa, expression of P-selectin, and an increased procoagulant activity (Peerschke, et al, 1993).

Another protein known to mediate platelet binding to the subendothelium is vWF (Stel, et al, 1985). vWF is a multimeric protein ranging in size between 500 and 20 000 kDa, but only the larger multimers are hemostatically active (Furlan, 1996). Most materials in contact with human plasma will adsorb some vWF (Horbett and Counts, 1984). A variety of responses have been shown by platelets after binding to vWF. vWF has been

shown to increase the intracellular calcium levels in platelets (Ikeda, et al, 1993), induce platelet aggregation (Minamoto, et al, 1996), promote formation of thrombi (Lambrecht, et al, 1986), induce actin polymerisation (Yuan, et al, 1999), and generate a high procoagulant activity of the platelet surface (Grunkemeier, et al, 2000a).

Fibronectin and vitronectin adsorbed to surfaces have been shown to promote platelet adhesion (Houdijk, et al, 1986; Tsai and Horbett, 1999) but have very little effect on the procoagulant activity of the platelets (Grunkemeier, et al, 2000b), a similar effect as obtained by surface bound fibrinogen.

The role of platelets at biomaterial surfaces

Not much is known about the role of platelets at biomaterial surfaces. In the use of extracorporeal circuits and vascular grafts, the formation of thrombi is of high concern. Heparin and hirudin among other platelet and coagulation inhibitors, are often used to minimise this formation, which could result in other bleeding complications and platelet dysfunction (Edmunds, 1985).

Platelet derived microparticles expose a very procoagulant surface (Pasquet, et al, 1996; Tans, et al, 1991; Thiagarajan and Tait, 1991) and associate with fibrin during thrombosis (Siljander, et al, 1996). Microparticles have also been shown to bind, activate, and aggregate neutrophils (Jy, et al, 1995), and materials promoting formation of microparticles (Gemmell, et al, 1995) can generate systemic effects.

When using bone anchored and subcutaneous biomaterials the setting is different. This situation is more like the healing of a normal wound, with the extra element of a biomaterial surface. As for normal wound healing, the healing process starts with coagulation and clot formation, but in this case around the implant. Inflammatory mediators are released and leukocytes are recruited to the site. Since this is a chain of reactions leading to either healing or rejection of the implant, every step of the way is of importance and to some degree governed by the previous.

As described above, platelets show a variety of responses to different proteins adsorbed to surfaces, transferring these signals to other cells. New platelets are recruited by release of platelet activating mediators such as serotonin (Li, et al, 1997), and by exposure of adhesion molecules such as fibrinogen and vWF (Tan, et al, 1999). Leukocyte rections are affected by release of soluble mediators (Holmsen and Weiss, 1979) and exposure of P-selectin (Yeo, et al, 1994).

The initial adhesion of platelets at hydrophobic titanium surfaces has been shown to exceed the adhesion seen on hydrophilic titanium surfaces (Nygren, 1996). The next step in the non-self recognition of these two biomaterials, the adhesion of neutrophils, has been shown to be mediated by two different receptors. At the hydrophobic titanium surface neutrophil adhesion can be inhibited by antibodies against PSGL-1, indicating that this initial adhesion is to platelets exposing P-selectin, while on the hydrophilic surface the adhesion appears to be mediated by IgG (Eriksson and Nygren, 2001b).

Aims of the present thesis

- to elucidate the kinetics of platelet adhesion and activation at hydrophilic and hydrophobic model surfaces and to investigate the thrombin dependence of the studied events
- to develop a new method for separation of platelets using a reversible inhibitor of platelet activation
- to investigate the activation of platelets and identify key proteins responsible for different parts of the platelet response at biomaterial surfaces
- to identify the primary adhesion molecule on platelets responsible for initial platelet adhesion to biomaterials when in contact with whole blood
- to investigate the effects of soluble signals from plasma proteins on fibrinogen adherent platelets

Experimental methodology

A potted version of materials and methods used in Paper I-V is given below. For a more detailed description the reader is referred to the individual Papers. A characterisation of the rabbit serum used in Paper IV and V is also given.

Surface preparation and analysis

Preparation of hydrophilic and hydrophobic glass slides and tubes

Hydrophilic and hydrophobic (methylated silica) slides and tubes were prepared by cleaning ordinary microscopy slides (80% SiO_2) or the inner surface of small glass test tubes (SiO_2 content 72-74%, 6 mm ∅) for 30 minutes in 70% ethanol containing 0.35 M HCl. After washing and drying the slides and tubes were hydrophilic. To make the slides and tubes hydrophobic, methylisation of the surfaces were performed by incubation in 0.1% 1,1,1,3,3,3-hexamethylsilazane in dichloromethane for 5 minutes followed by drying. The contact angles for the microscopy slides prepared above were $< 10°$ and $> 85°$ respectively, as indicated by measurement of saline drops (Dahlgren and Sunqvist, 1981).

Surface adsorption of plasma proteins

In Papers II-V protein solutions were physically adsorbed to hydrophobic glass surfaces. Fibrinogen, human albumin, vWF, and human IgG, were dissolved in D-PBS to a concentration of 100 µg/ml. Human thrombin was dissolved in D-PBS to a concentration of 240 µg/ml.

Lyophilised platelet-poor normal plasma and plasma with factor VIII deficiency were rehydrated and allowed to stand for at least 10 minutes before use. According to the manufacturer's specification, citrate was used as anticoagulant at the time of preparation. In Paper III, $CaCl_2$ was at times added to the plasma solutions to compensate for the citrate, before surface adsorption. This resulted in coagulation of the normal plasma during the adsorption procedure.

Drops (50 µl) of each plasma and protein solution were incubated on hydrophilic and hydrophobic slides in a humid chamber at room temperature for 15 or 30 minutes

respectively. For each tube a volume of 220 µl of the plasmas or protein solutions were used. The surfaces were then rinsed with D-PBS and used within an hour of preparation.

Ellipsometry

The optical density of physically adsorbed protein layers were analysed with an isoscope ellipsometer (Stenberg and Nygren, 1983). Quartz (SiO_2) on silica was washed and prepared as described above generating hydrophilic and hydrophobic surfaces. Proteins and plasmas were incubated on the surfaces in a humid chamber as described above. After rinsing with D-PBS and distilled water followed by drying with an air current, the optical density of the adsorbed protein films (Å) were determined using a quartz reference system. Very small intra protein variations were seen. In Paper V the protein concentration was calculated from the molecular refractivity of albumin (Stenberg and Nygren, 1983). No assumptions about functionality of the proteins were made as the intention was to resemble a biomaterial surface introduced into the body.
The enzyme activity of the thrombin after coating was checked using S-2258 (Chromogenix, Mölndal, Sweden) (Nydahl, et al, 1992), indicating that the adsorbed thrombin retained its enzymatic activity during the experiments.

Platelet isolation and characterisation

Preparation of platelets

In Paper II a new method for preparation of platelets was developed from Braide and Bjursten's method for leukocyte preparation (Braide and Bjursten, 1986). This method was used in subsequent Papers. Venous blood from healthy human donors, not on medication, was collected into citrate-phosphate-dextrose anticoagulant. To prevent platelet activation, 4 mM magnesium ions (Gawaz, et al, 1994) was used throughout the isolation procedure. The interchange between magnesium and calcium ions binding in certain integrins, especially GPIIb/IIIa (Gulino, et al, 1992; Ginsberg, et al, 1993), gives the possibility of a reversible inhibition of platelet activation during separation.
The platelets were separated by centrifugation over a Percoll gradient of three layers with different density and osmolarity. After washing, the platelets were resuspended and pooled in D-PBS containing albumin (1 mg/ml, pH 7.4). The platelet concentration was determined by counting in a Bürker chamber.

Flow cytometry, viability testing, and transmission electron microscopy

In order to characterise the platelets after the isolation, flow cytometry, viability staining, and TEM were performed in Paper II. The number of cells, classified by differences in size, were counted using flow cytometry according to standard procedures at a local hospital. The number of both leukocytes and erythrocytes were below countable concentrations for all preparations. During the routine counting procedure in a Bürker chamber, occasional leukocytes were detected. The number of leukocytes was however so small that any effects of leukocytes could be neglected and is disregarded in all Papers.

For viability and enzyme functionality testing of the platelets, different preparations of platelets were stained with FDA (Jones and Senft, 1985). The non-polar ester FDA, passes through cell membranes and is hydrolysed by intracellular esterases to produce fluorescein, a polar substance that passes slowly over the cell membrane (Rotman and Papermaster, 1966). The platelets showed a green fluorescence, thus having an intact intracellular esterase pool.

The morphology of the platelets was investigated using TEM. The isolated platelets were fixed and embedded in Epon plastic according to routine procedures. The specimens were sectioned and examined in a Philips TEM. The isolated platelets were morphologically intact with some cytoplasmic processes and intracellular canaliculi. The platelets contained both α- and dense granules as well as mitochondria of normal size.

Blood and platelet exposure to surfaces

Exposure of hydrophilic and hydrophobic glass to blood

In Paper I venous blood from healthy donors was drawn directly into a syringe (5 ml) containing 0.2 ml D-PBS, heparin (final conc. 10 IU/ml), or hirudin (final conc. 50 IU/ml).

The blood was placed in drops of approximately 100 µl on slides in a humid chamber at 37 °C. The exposure time varied from 5 seconds through 8 minutes. The blood was then washed off by rinsing the surface with D-PBS. The samples were fixed in 1% formaldehyde for 15 minutes at room temperature and rinsed before incubation with antibodies.

Adhesion and activation of isolated platelets

The coated slides were incubated with platelets (2,5 x 10^7 platelets/ml) for 1 to 30 minutes in a humid chamber at 37°C. In Paper II the effect of soluble vWF was investigated by adding pure vWF at a concentration of 10 µg/ml to the platelet suspension during incubation. The samples were washed with D-PBS and fixed in -20°C ethanol for 10 minutes (Paper II) or 1% paraformaldehyde in D-PBS for 15 minutes at room temperature. After fixation the samples were rehydrated and/or washed with D-PBS before antibody staining. No differences in antibody staining were seen between the fixation methods used.

In Paper V some slides were after incubation gently rinsed and exposed to different plasma proteins for 5 minutes at 37°C in a humid chamber. The protein solutions used were; D-PBS (control), IgG, thrombin, and vWF. The samples were washed with D-PBS before fixation and staining.

Inhibition of platelet binding with antibodies

In Paper IV inhibition studies of early platelet binding to surfaces were performed. Antibodies against different surface antigens on platelets, presented in Table II, were diluted to a final concentration of 10 µg/ml. Control experiments were performed using D-PBS. The blood mixed with antibodies was incubated on preheated (37°C) hydrophilic and hydrophobic slides for 2 minutes. The samples were then washed with D-PBS before fixation and staining.

Table II

Surface receptors	Other names	Antibodies used
CD9		MM2/57 and 4E1
FcγII-receptor	CD32	AT10
integrin α_{IIb}	CD41, GPIIb	M148 and SZ22
integrin α_5	CD42b, GPIb	AN51
integrin α_v	CD51, VNRα	AMF7
integrin β_3	CD61, GPIIIa	C17 and SZ21

Inhibition experiments were also performed for the CD41a antibody clone M148 at final concentrations of 100 and 50 ng/ml.

Control experiments for antibody inhibition

To test the receptor inhibiting effect of the antibodies used in Paper IV, control experiments were performed using isolated platelets. The platelet solution was diluted to a final concentration of 5 x 10^7 platelets/ml after addition of antibody (final conc. 10 µg/ml). Comparative experiments were always performed at the same time using D-PBS. Drops (40 µl) were transferred to surfaces coated with corresponding adhesion protein (when known) and one or more control proteins. The solution was incubated for 5 minutes in a humid chamber at 37°C.

A series of dilutions were also done for the CD41a antibody clone M148, with the lowest concentration being 40 ng/ml. This was done to achieve specific inhibition of platelet binding to fibrinogen binding but not to IgG. The samples were washed with D-PBS before fixation and staining.

Staining and analysis

Immunofluorescence

The number of adhering platelets, coverage, morphology, spreading, and exposure of CD62P and vWF were determined using immunocytochemistry. For platelet staining in Paper I-III pan platelet antibodies preadsorbed with human platelet poor plasma were used. In Paper IV and V rabbit serum, produced and characterised as described below, against human platelets were used. Exposure of CD62P and binding of vWF on the surface were determined using monoclonal antibodies. The samples were incubated for 30 minutes with the primary antibody or anti-serum on a cooling plate, 0°C, (HISTO-LAB, Sweden) followed by rinsing with D-PBS before a second incubation with FITC or TRITC-conjugated secondary antibodies. The double labelling in Paper I and V were performed by subsequent incubations of the antibodies. After rinsing the samples were assembled with 1,4-diaza-bicyclo[2,2,2]octane mixed with glycerol to keep the fluorescence from fading. Viewing and photography were done within 6 hours.

Analysis of immunoflourescence

All samples were photographed in a Zeiss 3RS fluorescence microscope, using Kodak 400 film (Paper I-III) or CCD camera (Paper IV and V). Two or three photos were taken on each protein or plasma spot.

After development the films were scanned and the coverage, the spreading and the number of platelets adhered to the surface were analysed in Adobe Photoshop or NIH Image. The area for analyse was kept at 70 000 μm^2.

In Paper I and II a spreading index (SPI) for the platelets was calculated as the surface coverage divided by the number of platelets (multiplied by 100). For compact platelets the SPI was about 1.5 and for fully spread platelets the SPI was about 12.2. In the other Papers the actual area per stained platelet was calculated instead, corresponding to the SPI multiplied by 7.2.

The normalised surface coverage of vWF in Paper I and of CD62P in Paper V was calculated as coverage of vWF or CD62P divided by the total coverage of platelets, while the per cent of CD62P exposure in Paper III was calculated as the number of CD62P positive platelets divided by the total number of platelets at the surface.

Analysis of secreted products

Platelets at a concentration of 10^7-10^8 platelets/ml were incubated in plasma or protein coated tubes for 10 minutes at 37°C. The tubes were tcentrifuged and the supernatants were collected for analysis of secreted products. For consecutive signalling in Paper V, fibrinogen coated tubes were instead rinsed once with D-PBS (37°C) after the platelet incubation, before a secondary incubation (5 min, 37°C) with protein solutions. The solutions used were: D-PBS (control), IgG, thrombin, and vWF. These tubes were also centrifuged and the supernatants were collected.

The concentration of PF4 (1 x 10^7 platelets/ml or 2.5 x 10^7 for consecutive signalling) was determined from samples (frozen less than a month) by a commercially available kit (Asserchrom, Diagnostica Stago) according to manufacturer's procedure. PF4 was used to detect release of α-granules. The low concentration of platelets used (1 x 10^7 platelets/ml) was to avoid overload of the enzyme kit.

For detection of dense granular release, the concentration of ADP and ATP (2,5 x 10^7 platelets/ml) were determined. ADP was converted to ATP by a method described by Feraudi et al (1981). The supernatants were mixed with an enzyme solution containing creatine phosphokinase and phosphocreatine. The samples were incubated for 30 minutes at 37°C before the reaction was stopped by incubation at 80°C for 5 minutes. The enzyme activity, as conversion factor for ADP to ATP, was determined for every experiment (ADP conc. 10^{-6}-10^{-8} M) and used to calculate the actual concentration of ADP released.

The concentration of ATP was determined by a commercially available kit (ATP Bioluminescence Assay Kit CLS II, Boehringer Mannheim GmbH, Germany) according to manufacturer's procedure.

Phospholipids (10^8 platelets/ml) in the supernatant in Paper III were determined using HPTLC after acidification of the supernatant. The phospholipids were extracted with 1-butanol and washed with water before evaporation (Eichholtz, et al, 1993). 1-butanol was chosen as extraction medium to achieve as high recovery as possible of polar lipids (Bjerve, et al, 1974; Kolarovic and Fournier, 1986). The residues were dissolved in $CHCl_3$:MeOH:Hac (19:1:1). Samples (5 µl) were applied on precoated silica gel 60 plates. LPA was used as a phospholipid standard. The HPTLC plates were developed by $CHCl_3$:MeOH:7M NH_3 (aq) (12:12:1). After drying, all phospholipids were detected by using post-chromatographic derivatisation by manganese (II)-sulphuric acid reagent followed by heating (120°C for 30 min). Phospholipid fractions ≥ 200 ng appeared as brown zones. This is a coarse method for lipid detection and only high levels of phospholipids could be detected.

Blotting procedure

Isolation of platelet membrane proteins

Venous blood from healthy human donors not on medication was anticoagulated with EDTA. The platelet membrane proteins were isolated according to the procedure described by Fitzgerald et al (1985). In short, PRP was collected after centrifugation (15 min, 300 g). The PRP was transferred to new tubes and centrifuged at 300 g for 5 minutes to remove all red blood cells. This procedure was repeated until no red blood cells were evident in the pellets. The PRP was centrifuged (15 min at 1800 g) to pellet the platelets. The platelets were washed three times in TBS (20 mM Tris – HCl and 150 mM NaCl, pH 7.4) containing EDTA (1 mM) before they were lysed by resuspension in TBS containing 1% Triton X-100, 1 mM $CaCl_2$, and 0.1 µM leupeptin hydrochloride. The lysate was ultra centrifuged at 20 000 g for 15 minutes at 4°C to remove cytoskeletal elements and stored at -20°C until use.

Immunoblotting

The platelet lysate was diluted 1:5 before electrophoresis on 4-12% Bis-Tris Gel (NuPAGE) run with MOPS running buffer (200 V, 45 min) under non-reduced

conditions. The gels were blotted (30 V, 1.5 h) onto nitrocellulose membranes (Invitrogen). As molecular weight standard MultiMark™ was used. The membranes were blocked in TBS containing Tween 20 (0.1%), non fat milk (5%), and sodium azide (0.02%) for 2 hours. After rinsing, the membranes were incubated over night with M148, C17, SZ21, or/and SZ22 antibodies, followed by rinsing and incubation with secondary peroxidase conjugated anti-mouse antibodies. After thorough rinsing, an agarose gel (40°C) containing 0.01% H_2O_2 and 1 mg/ml paraphenylene diamine was poured over the membrane. After 5 minutes the gel was removed and the binding of antibodies could be seen as dark bands.

Statistical evaluations

Statistical evaluations were made using Student's t-test (paired when possible) in Papers I-III and V. In Paper IV, Z-test, as well as paired Student's t-test, was performed on the inhibition data. The value for significance was set to $p < 0.05$.

Production and characterisation of rabbit serum

Production of rabbit serum against human platelets

For platelet staining in Paper IV, rabbit serum against human platelet was used. The serum was produced by injecting rabbits 5 times with human platelets (10^7) mixed with Freunds incomplete adjuvant, followed by terminal bleeding. The immunisation was approved by the ethical committee of the University of Göteborg. After coagulation the blood was centrifuged (1500g, 15 min) and the serum collected. The anti-serum was incubated overnight with platelet free human plasma (4°C) before centrifugation (2500 g, 10 min) to get rid of binding to human plasma proteins.

Gel electrophoresis and immunoblotting

The platelet lysate, prepared as described above, was electrophoresed using 4-12% Bis-Tris Gel run with MOPS running buffer under both reduced and non-reduced conditions according to manufacturer's procedure. Some gels were stained with Coomassie blue overnight (0.03%, 50% methanol, 10% acetic acid, 40% H_2O) and destained until the bands were clearly separated before scanning with a colour scanner (UMAX Astra 2400S, Umax Data Systems Inc., Taiwan). The stained gels of platelet lysates is shown in Figure 2.

Other gels were blotted onto nitrocellulose membranes. The membranes were incubated with rabbit anti-human platelet serum followed by peroxidase conjugated anti-rabbit antibodies. The binding of antibodies could be seen as dark bands (Figure 3). As shown in the figure, the rabbit anti-human platelet serum recognised a variety of platelet membrane proteins of different sizes.

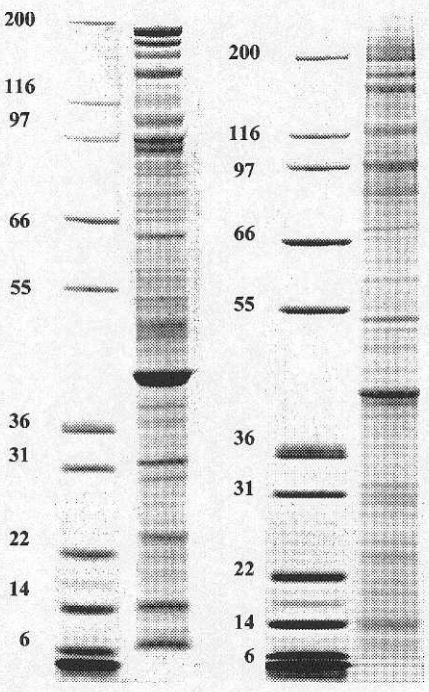

Figure 2. Gel electrophoresis of platelet lysates under reduced and non-reduced conditions, run on a 4-12% Bis-Tris Gel with MOPS running buffer.

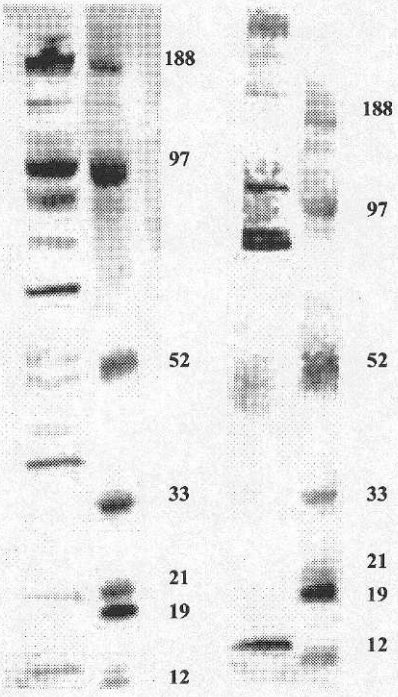

Figure 3. Western blots of the platelet lysates shown in Figure 2, using rabbit anti-human platelet serum.

Summary of results

Paper I

This Paper investigates the initial interaction of platelets with two model surfaces, hydrophilic and hydrophobic glass. The kinetics of platelet adhesion differed between the two surfaces, showing a peek in adhesion after half a minute on the hydrophilic surface whereas on the hydrophobic surface a constant increase during the incubation time was seen. On the hydrophilic surface, binding of vWF and exposure of CD62P, both normalised to platelet coverage, were high after 8 minutes (Table III). To investigate the thrombin dependency of this activation, venous blood was anticoagulated with heparin or hirudin, resulting in a low normalised binding of vWF and exposure of CD62P on both surfaces.

Table III

	vWF	CD62P
Hydrophilic glass		
untreated blood	0.17 ± 0.03	0.18 ± 0.04
heparinised blood	0.04 ± 0.01	0.03 ± 0.00
hirudinised blood	0.07 ± 0.02	0.00 ± 0.00
Hydrophobic glass		
untreated blood	0.03 ± 0.02	0.03 ± 0.00
heparinised blood	0.03 ± 0.01	0.01 ± 0.00
hirudinised blood	0.00 ± 0.00	0.01 ± 0.00

However the number of platelets adhering to the surfaces increased with both anti-coagulants compared to untreated blood. The spreading of surface adherent platelets was inhibited by heparin, but not hirudin, showing that this was not a thrombin dependent part of platelet activation.

Paper II

To be able to more specifically study platelet reactions at surfaces, a new fast and easy method for isolation of platelets was developed. The use of magnesium to prevent

activation of platelets during separation was clearly reversible though a complete inhibition was not seen. After resuspension in D-PBS containing calcium, the platelets could be stored at room temperature for hours without formation of aggregates.

To investigate the functionality of the platelets, isolated platelets were allowed to adhere to protein coated surfaces. On the IgG coated surface, platelets formed a variety of different morphologies. After about 10 minutes, fragmentation of platelets was seen, leaving surface bound microparticles. Platelets forming long filipodial extensions with microparticles along were also seen (Figure 4). At the same time an increase of adhesion kinetics was also observed.

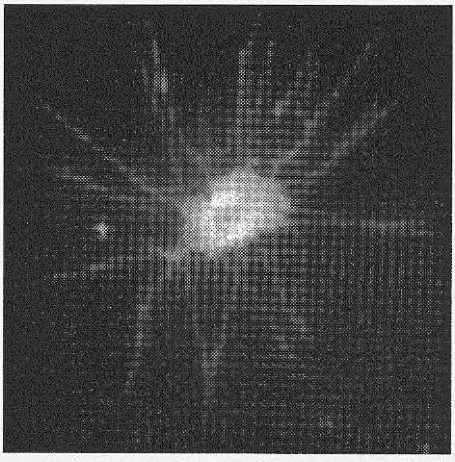

Figure 4. A single platelet adhered to an IgG coated surface forming filopodial extensions with surface bound microparticles along them. The diameter of the central body is 3 µm.

The exposure of CD62P on the platelet surface was also investigated. On the albumin coated surface the lowest level of CD62P exposure was seen. Only about 11% of the adhered platelets exposed CD62P. Any platelet activated during the isolation process would probably have an increased binding capacity and accumulate at the surface. On the surface coated with normal human plasma, 67% of the platelets exposed CD62P while on the surface coated with factor VIII deficient plasma only 28% of the platelets exposed CD62P, indicating a possibility of partial activation of the platelets as an effect of single proteins in the coating.

Paper III

This Paper is an extension of the findings of the effect of coagulation factor VIII in Paper II. The effect of vWF was investigated using both the pure protein and factor VIII deficient plasma. Factor VIII deficient plasma lacks all parts of coagulation factor VIII, including vWF.

The differences in adhesion and spreading were not very large. However, the morphology of the platelets on the surfaces differed, showing a higher degree of filipodial formations on the surfaces where vWF was present. The main finding in this

Paper is the dependency of vWF for the exposure of CD62P on the platelet surface (Figure 5). Even with calcium present at the time of physical adsorption, the percentage of platelets exposing CD62P did not reach 40% on the surface coated with factor VIII deficient plasma.

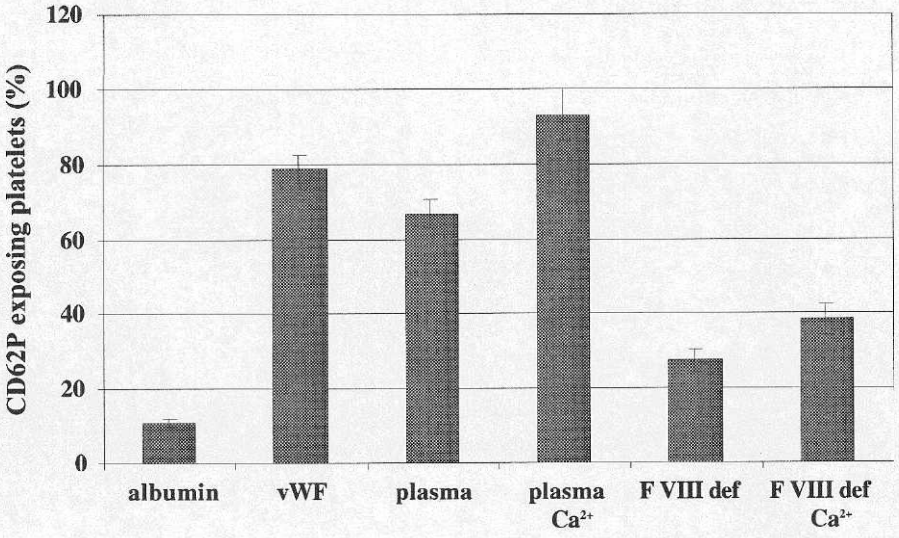

Figure 5. Percentage of platelets on different coatings exposing CD62P. F VIII def = Factor VIII deficient plasma. Ca^{2+} = Calcium ions present at the time of physical adsorption. Mean and standard error of mean is given (n = 9).

The release products analysed from the supernatant of incubated platelets (ATP, PF4, and phospholipids) were rather low and did not differ between the surfaces, showing that platelets are capable of mediating variable responses, not detonating as hand-grenades, which they have previously been compared to.

Paper IV

In Paper IV the initial binding of platelets to hydrophilic and hydrophobic glass was investigated using whole blood. Different antibodies were used to investigate platelet adhesion. Most antibodies were found to inhibit the binding capacity of their corresponding integrin.

The only antibody generating a very high decrease in binding to the surfaces is known to immunoprecipitate the glycoprotein IIb/IIIa (Figure 6). Antibodies directed to the different integrin chains of GPIIb/IIIa showed no or very little inhibiting effect. A slight

inhibition was seen for one of the CD9 antibodies (MM2/57) on the hydrophobic surface, while the other CD9 antibody (4E1) increased platelet binding to the hydrophilic surface. Some of the other antibodies also generated an increase in platelet binding, especially on the hydrophilic surface, indicating platelet activation.

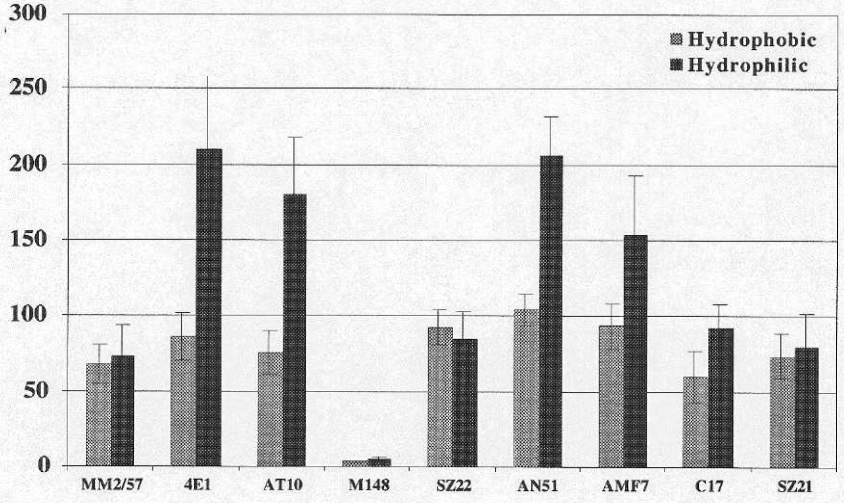

Figure 6. Platelet adhesion from whole blood after incubation for 2 minutes on hydrophilic or hydrophobic glass with different antibodies present. Values are given as percentage adhesion compared to control. Antibodies MM2 and 4E1 are directed against CD9, AT10 against FcγII-receptor (CD32), M148 and SZ22 against GPIIb (CD41, integrin α_{2b}), AN51 against GPIb$_\alpha$ (CD42b, integrin α_5), AMF7 against integrin α_v (CD51), and C17 and SZ21 against GPIIIa (CD61, integrin β_3). Mean and standard error of mean is given (n = 6).

One of the antibodies against GPIIb/IIIa used (SZ21), with no effect on platelet adhesion, is known to inhibit fibrinogen mediated aggregation. This clearly indicates two different fibrinogen binding parts of GPIIb/IIIa, where only one is important for the initial platelet adhesion to surfaces.

Paper V

In this Paper the platelet responses after single and consecutive exposure to plasma proteins were compared.

Platelets adhered to the fibrinogen coated surface were seen to show the significantly lowest release of α-granules (Table IV), both detected by normalised exposure of CD62P and by released PF4. However, platelets adhered to fibrinogen spread on the surface, but when thrombin was added to the fibrinogen-adherent platelets they decreased their spreading significantly. All protein coating except fibrinogen were seen to expose CD62P on the surface and release PF4. The significantly highest release of PF4 was seen on the IgG coated surface.

Table IV

	Spreading μm^2/platelet	**CD62P** % normalised	**PF4 release** IU/ml
Single exposure			
fibrinogen coating	27.0 ± 1.6	13.3 ± 1.9	50.4 ± 4.6
IgG coating	27.5 ± 1.1	21.2 ± 1.3	105.0 ± 5.5
thrombin coating	28.5 ± 1.2	20.0 ± 2.1	88.1 ± 4.7
vWF coating	24.3 ± 0.9	25.4 ± 2.2	70.3 ± 4.9
Consecutive exposure			
D-PBS (control)	30.2 ± 1.6	11.3 ± 1.7	0.8 ± 0.2
IgG	27.9 ± 1.3	13.5 ± 2.5	1.1 ± 0.3
thrombin	25.1 ± 1.2	22.9 ± 3.5	16.2 ± 3.2
vWF	29.0 ± 1.0	12.9 ± 1.1	1.2 ± 0.4

When fibrinogen-adherent platelets were exposed to soluble plasma proteins only thrombin was shown to have any significant effect. The exposure of CD62P and the release of PF4 were very small for the incubation of fibrinogen-adherent platelets with D-PBS (control).

As for the release of α-granules, the highest concentration of both ATP and ADP were seen after incubation of fibrinogen-adherent platelets with thrombin (Figure 7).

Thrombin coating was also seen to generate the highest concentration of both ATP and ADP.

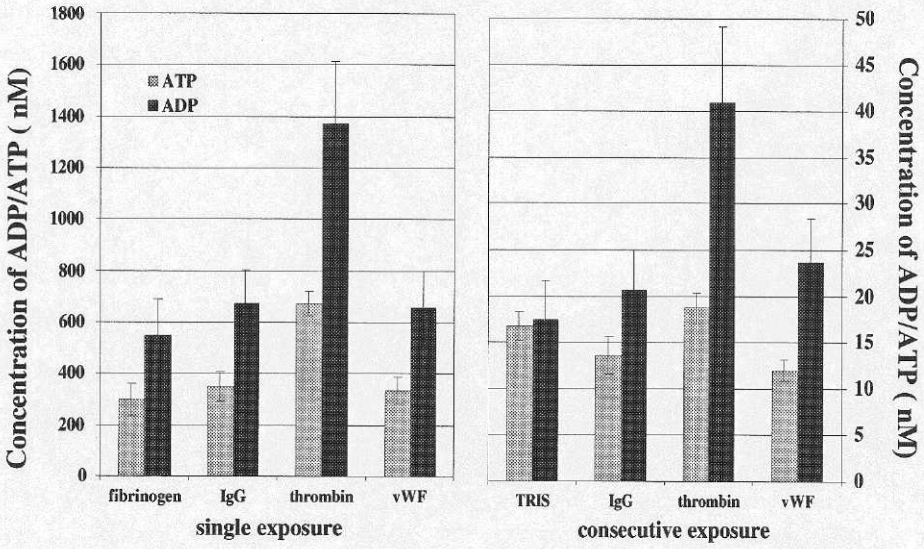

Figure 7. Concentrations of ATP and ADP after single and consecutive exposure to plasma proteins. Mean and standard error of mean is given ($n \geq 10$).

As shown in this Paper all these plasma proteins are potent mediators, either as surface bound, or as soluble. The effect of these proteins are dependent on previous events of the platelets, as for example adhesion to fibrinogen.

Discussion

Platelets, perhaps because of their size and definition as being cell fragments, are often omitted in the discussion regarding healing around implants. The role of platelets in inflammatory responses are however substantial. The probability of a cell or a cell fragment with such a huge functionality being present at a site without influencing the outcome is minimal.

For better understanding of the role of platelets in the healing at biomaterial surfaces, both whole blood and a reduced system were used in this thesis. In reduced systems, the effect of proteins on platelet adhesion and reactions can be studied by two different strategies, either by coating of single protein solutions, or by coating with deficient plasmas. Both ways have their advantages and disadvantages. When using single proteins the protein film formed is easily defined, but a higher concentration of the protein on the surface compared to the *in vivo* situation is usually the outcome. Using deficient plasmas can result in changes in adsorption patterns, but a situation more close to *in vivo* is generally achieved as many early interactions are still present. In Paper III both methods were used to elucidate the role of surface bound vWF and the results were seen to coincide.

Another approach for better understanding of the complicated interactions at biomaterial surfaces is the use of inhibitors and anticoagulants. In Paper I, both heparin and hirudin were used. Both the exposure of CD62P and the binding of vWF were found to be thrombin dependent events since they were inhibited by hirudin. These events mainly took place on the hydrophilic surface where more thrombin is generated through surface activation of the coagulation system (Vroman, et al, 1980). Heparin, a multi-potent inhibitor (Engstad, et al, 1997; Horne and Chao, 1989; Sobel, et al, 1996), was seen to inhibit platelet spreading as well as the exposure of CD62P and binding of vWF.

The use of antibodies to inhibit platelet interactions is specific but controversial since many antibodies are known to elicit an activation of platelets (Anderson, et al, 1991; Kuroda, et al, 1995; Modderman, et al, 1988). The antibodies used in Paper IV where tested in the reduced system to verify their inhibitory effect on corresponding receptors and to detect any signs of activation. In Paper I, exposure of CD62P on the platelet surface was found to take approximately 8 minutes of blood – material contact on the same surfaces as used in Paper IV. This indicates a longer platelet activation time than

the incubation times used in Paper IV. However, on the hydrophilic surface, an increase in platelet adhesion was seen for some antibodies, suggesting that this could be either an effect of concentration / conformation of fibrinogen on the surface (Chinn, et al, 1991; Nygren, et al, 1992; Nygren, et al, 1994), or a thrombin dependent effect (Vroman, et al, 1980).

The adsorption of fibrinogen to biomaterial surfaces has long been known to increase platelet adhesion (Packham, et al, 1969; Zucker and Vroman, 1969; Mason, et al, 1971), and the most abundant receptor on the platelet surface is the fibrinogen receptor, GPIIb/IIIa (McEver, et al, 1983). This receptor has two different fibrinogen binding sites, dependent on the two different activation states of this integrin (Coller, 1980; Savage and Ruggeri, 1991). In Paper IV, the initial platelet adhesion to both hydrophilic and hydrophobic surfaces was shown to be mediated by the non-activated form of GPIIb/IIIa. As thrombin is known to activate GPIIb/IIIa (Minckes, et al, 1994), one might suggest that platelet binding to hydrophilic surfaces is partly mediated by the activated state of GPIIb/IIIa. However, antibodies known to inhibit fibrinogen mediated aggregation, e.g. the activated state of GPIIb/IIIa, did not have any inhibitory effect on platelet adhesion during the first two minutes of blood – material contact. Longer times were not investigated. However, it was shown in Paper I, and has been shown by others (Eriksson and Nygren, 2001a), that platelet coverage do not increase above 5% when no shear stress is present.

There are a number of different methods for separation of platelets. Most inhibitors of platelet activation prevent thrombin formation. Others, like prostacyclin and cAMP, have intracellular effects and act at levels secondary to priming (Colman, 1994, Pineo and Hull, 1999). Some of these inhibitors have been shown not to be able to prevent the initial adhesion of platelet to immobilised fibrinogen (Haimovich, et al, 1993), which is known to generate intracellular phosphorylations (Haimovich, et al, 1996a). In this thesis, the prevention of platelet activation was endeavoured in an earlier phase, extracellularly, prior to the generation of intracellular second messengers (Haimovich, et al, 1993, Haimovich, et al, 1996b). The platelet integrin GPIIb/IIIa has been shown to be inhibited by high levels of magnesium ions (Gawaz, et al, 1994), resulting in inhibited platelet spreading and decreased platelet activation. The inhibition is achieved by substi-tution of calcium ions in the integrins, especially in GPIIb/IIIa, with magnesium ions, thus preventing integrin functionality (Ginsberg, et al; 1993, Gulino, et

al, 1992). The substitution is reversible due to interchanges between magnesium and calcium ions (Gulino, et al, 1992). Clinical tests using elevated levels of magnesium ions to suppress platelet activation have shown a reduced platelet activation and prolonged bleeding times (Gawaz, et al, 1996; Ravn, et al, 1996).

Another possibility to inhibit GPIIb/IIIa is by Fab fragments of inhibitory monoclonal antibodies. Abciximab (Reopro) is a new platelet inhibitor, approved by the Food and Drug Administration. This inhibitor also cross-reacts with the vitronectin receptor (Topol, et al, 1999), suggesting inhibition of the activated state of GPIIb/IIIa.

Even though some platelet activation was seen in the TEM picture after the separation procedure in Paper II, only 11% of the platelets were seen to expose CD62P on their surface after adhesion to an albumin coated surface. After 10 minutes of exposure to a surface coated with factor VIII deficient plasma, only 28% of the platelets exposed CD62P. These two figures should be compared with the 56% of platelets exposing CD62P in suspension after an isolation procedure used by Frojmovic et al (1997). Activated platelets would probably have accumulated on the surface during the incubation time in Paper II since their adhesion receptors are active. Also, as CD62P function as a ligand for adhesion of neutrophils (Larsen, et al, 1989), any redistribution of the selectin (Escolar and White, 2000) would be to the surface exposed to antibodies, further implicating a low degree of activation for the separated platelets used in this thesis.

The use of isolated cells in experiments have many advantages but also drawbacks. By omitting other cells the reactions of interest could clearly be studied in small steps without interactions and reactions from other cells responding to the same stimuli. Changes are easier to monitor as no other interfering processes take place. This gives the opportunity to understand a small part of the complex biological system. The drawbacks are, apart from activation during the separation procedure, that the hemostatic system is set off balance (Escolar and White, 2000). One important consideration during the use of isolated systems is that the interactions studied must be performed in the time order and sequence they occur in the clinical situation. Such studies add specific knowledge to the field, even though a direct interpretation in the clinical situation is not possible.

From the separated system used in this thesis, it was possible to show that platelets respond differently to different proteins. In Paper III, vWF coated surfaces were seen to

generate a high exposure of CD62P on the platelet surface. This high exposure of CD62P is not surprising, as platelet adhesion to injured vessel walls leads to P-selectin exposure on the platelets (Andrews, et al, 1997). This exposure of P-selectin generates adhesion and accumulation of neutrophils at the site of vascular rupture (Diacovo, et al, 1996a; Diacovo, et al, 1996b). The same adhesion of neutrophils to platelets, mediated by PSGL-1, can be seen on hydrophobic titanium surfaces (Eriksson and Nygren, 2001b).

In Paper II, immobilised IgG was seen to generate a morphological response in platelets. Formation of long cytoplasmatic pseudopods, generation of microparticles, and disruption of the platelets were seen. These morphological changes generating microparticles are similar to platelet changes during certain forms of thrombocytopenia (Warkentin, 1996) and this reaction has been suggested to be generated by immune complexes (Goad, et al, 1994; Leir, et al, 1995). The generation of microparticles is of biological importance. Microparticles accumulate procoagulant activity (Siljander, et al, 1996) and can be transported throughout the body by the blood stream (Gemmell, et al, 1995), causing systemic effects. Similar platelet morphologies with generation of long pseudopods, have been seen after adhesion of isolated platelets to uncoated polymeric materials (Waples, et al, 1996).

The healing process at a biomaterial surface is comparable to the healing process of a normal wound, but modified by protein and cell reactions at the implanted material. Upon vascular rupture the first reaction for platelets are binding to extra-cellular matrix, or plasma proteins bound to the extra-cellular matrix. When a biomaterial is present at the site of an injury, platelet could also adhere to proteins adsorbed to the biomaterial. It was shown in Paper IV that the initial binding of platelets to biomaterial surfaces were mediated by GPIIb/IIIa, the fibrinogen receptor. This knowledge was used in Paper V to investigate platelet reactions to plasma proteins.

In the healing process, the secondary signals after platelet adhesion are of importance for the generation of platelet responses. In Paper V, a new model suitable for studies of such interactions was developed. After prior adhesion to fibrinogen, the adherent platelets were exposed to plasma proteins. Thrombin was found to be the most potent protein and a release of both α- and dense granules were found. This could affect the outcome of the healing process by attraction of more platelets as well as inflammatory cells to the area. IgG and vWF, which were shown in Papers II and III to have

pronounced effects on platelets when surface bound, had very little effect on the platelets as soluble mediators after fibrinogen adhesion.

Adhesion of platelets to fibrinogen is known to generate intracellular phosphorylation reactions (Haimovich, et al, 1996a) without generation of release reactions (Heemskerk, et al, 1997; Packham, et al, 1969) or procoagulant activity (Grunkemeier, et al, 2000). The phosphorylation of proteins after adhesion to fibrinogen might interfere with intracellular reactions generated by occupancy of other receptors. The focal adhesion kinase $pp125^{FAK}$ is known to be phosphorylated via protein kinase C activation both after fibrinogen binding to GPIIb/IIIa and IgG binding to the FcγII-receptor (Haimovich, et al, 1996b).

Most plasma proteins affecting platelet responses have more than one platelet receptor capable of binding the same ligand. The receptors often function differently. They may have different affinity constants (Greco, et al, 1996), work under different shear stress (Ikeda, et al, 1991), or differ in their requirement of pre-activation (Savage, et al, 1992). These dual activation pathways through different receptors give a possible explanation for the differences in responses seen between platelets adhering to a surface immobilised protein and adherent platelets exposed to the same protein in soluble form. However, the similarity to the process of normal wound healing remains, and the understanding of this process could be investigated further using specific blocking agents.

The understanding of every step in the healing process is of importance for the understanding of the whole process. For example, the adhesion of platelets to titanium is rather high (Kanagaraja, et al, 1996). The titanium surface has also been defined as highly thrombogenic (Hong, et al, 1999). This thrombogenicity of titanium might be the reason behind the great success in the use of this material for bone anchoring implants. If this is the case, platelet reactions at the implant surface is one of the most important events during the non-self recognition of surfaces.

This thesis provides information about the initial reactions of platelets in contact with biomaterial surfaces. This knowledge may be useful in the design of future biomaterials by indicating the role of platelets in blood – biomaterial interactions. The thesis also provides methods suitable for platelet separation and for investigation of the initial platelet reactions to biomaterial surfaces.

Conclusions

- The platelet kinetic differed between hydrophilic and hydrophobic surfaces both regarding adhesion and activation. A thrombin dependency was found for the exposure of CD62P as well as the binding of vWF.
- A new method for platelet isolation based on the leukocyte preparation method described by Braide and Bjursten (1984) was developed. A reversible inhibition of platelet integrins by excessive amount of magnesium ions during the isolation procedure was used and the platelets were functional after separation.
- IgG was assigned to be a key protein for the fragmentation of platelets and together with thrombin, a key protein for the release of both α- and dense granules. The exposure of CD62P on the platelet surface was however assigned to von Willebrands factor.
- The main adhesion of platelets from whole blood to both hydrophilic and hydrophobic surfaces was mediated via GPIIb/IIIa and was inhibited by antibodies from clone M148, but not by antibodies known to inhibit fibrinogen binding to GPIIb/IIIa after ADP and collagen induced aggregation.
- The initial adhesion of platelets to fibrinogen render changes in the platelets that influences later responses to plasma proteins, affecting primarily the release of α-granules.

Acknowledgements

First of all I would like to express my gratitude to my supervisor professor Håkan Nygren. Thank you for introducing me to a fascinating research field and the support given throughout these years.

My thanks to professors Keiko Funa, Jan-Olof Karlsson and associate professor Bengt R Johansson, for questions and comments at my pre-dissertation, leading to valuable discussions and some rethinking.

I would also like to address a number of thanks to all former, present, and temporary members of the research group for valuable and enjoyable discussions about everything from science to Monty Python. Special thanks to Ann, Christin, Herman, Noushin, and Per for encouragement and advice during this last period. Even more special thanks to Cecilia Eriksson, my co-author, co-worker, and discussion partner, who have given valuable criticism during the final preparation of this thesis, she know as she has been there.

Warm thanks to Laila, Barbro, and Eva for taking care of all the logistics.

Finally I would like to thank both my families, the Brobergs and the Thörnqvists. Thank you all for encouragement, support, and recreation, not forgetting all tasty Sunday dinners. Special thanks to Kristen for help with the linguistics in this thesis, and to Martin, who has made my time outside work the most important part of my life.

The financial support of the Faculty of Medicine, University of Göteborg, and the Swedish Research Council of Engineering Science is gratefully acknowledged.

References

Anderson GP, van de Winkel JG, Anderson CL. Anti-GPIIb/IIIa (CD41) monoclonal antibody-induced platelet activation requires Fc receptor-dependent cell-cell interaction. *Br J Haematol* 1991;79:75-83.

Andrews RK, Berndt MC. Adhesion-dependent signalling and the initiation of haemostasis and thrombosis. *Histol Histopathol* 1998;13:837-44.

Andrews RK, Fox JE. Interaction of purified actin-binding protein with the platelet membrane glycoprotein Ib-IX complex. *J Biol Chem* 1991;266:7144-7.

Andrews RK, Lopez JA, Berndt MC. Molecular mechanisms of platelet adhesion and activation. *Int J Biochem Cell Biol* 1997;29:91-105.

Aziz KA, Cawley JC, Treweeke AT, Zuzel M. Sequential potentiation and inhibition of PMN reactivity by maximally stimulated platelets. *J Leukoc Biol* 1997;61:322-8.

Baier RE, Dutton RC. Initial events in interactions of blood with a foreign surface. *J Biomed Mater Res* 1969;3:191-206.

Bazzoni G, Dejana E, Del Maschio A. Platelet-dependent modulation of neutrophil function. *Pharmacol Res* 1992;26:269-72.

Berndt MC, Mazurov AV, Vinogradov GF, Burns GF, Chesterman CN. Topographical Association of the Platelet Fc-receptor with the Glycoprotein IIb-IIIa Complex. *Platelets* 1993;4:190-6.

Berndt MC, Phillips DR. Purification and preliminary physicochemical characterization of human platelet membrane glycoprotein V. *J Biol Chem* 1981;256:59-65.

Bevers EM, Comfurius P, van Rijn JL, Hemker HC, Zwaal RF. Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. *Eur J Biochem* 1982;122:429-36.

Bic Z, Blix GG, Hopp HP, Leslie FM. In search of the ideal treatment for migraine headache. *Medical Hypotheses* 1998;50:1-7.

Bjerve KS, Daae LN, Bremer J. The selective loss of lysophospholipids in some commonly used lipid-extraction procedures. *Anal Biochem* 1974;58:238-45.

Blaas P, Berger B, Weber S, Peter HH, Hansch GM. Paroxysmal nocturnal hemoglobinuria. Enhanced stimulation of platelets by the terminal complement components is related to the lack of C8bp in the membrane. *J Immunol* 1988;140:3045-51.

Braide M, Bjursten LM. Optimized density gradient separation of leukocyte fractions from whole blood by adjustment of osmolarity. *J Immunol Methods* 1986;93:183-91.

Brandt E, Van Damme J, Flad H-D. Neutrophils can generate their activator neutrophil-activating peptide 2 by proteolytic cleavage of platelet-derived connective tissue-activating peptide III. *Cytokine* 1991;3:311-21.

Brisson C, Azorsa DO, Jennings LK, Moog S, Cazenave JP, Lanza F. Co-localization of CD9 and GPIIb-IIIa (alpha IIb beta 3 integrin) on activated platelet pseudopods and alpha-granule membranes. *Histochem J* 1997;29:153-65.

Broberg M, Nygren H. Platelet interaction with surface-immobilized IgG induces the secretion on surface bound microparticles and fragmentation of the cells. *Colloids and Surfaces B: Biointerfaces* 1998;10:251-62.

Brown MJ, Ind PW, Jenner DA. Platelet histamine. *New Engl J Med* 1980;303:756.

Buller HR, Ten Cate T. Coagulation and platelet activation pathways. A review of the key components and the way in which these can be manipulated. *Eur Heart J* 1995;16:8-10.

Castor CW, Walz DA, Ragsdale CG, Hossler PA, Smith EM, Bignall MC, Aaron BP, Mountjoy K. Connective tissue activation. XXXIII. Biologically active cleavage products of CTAP-III from human platelets. *Biochem Biophys Res Commun* 1989;163:1071-8.

Cazenave JP, Assimeh SN, Painter RH, Pachham MA, Mustard JF. Clq inhibition of the interaction of collagen with human platelets. *J Immunol* 1976;116:162-3.

Chacko GW, Duchemin AM, Coggeshall KM, Osborne JM, Brandt JT, Anderson CL. Clustering of the platelet Fc gamma receptor induces noncovalent association with the tyrosine kinase p72syk. *J Biol Chem* 1994;269:32435-40.

Chen M, Inestrosa NC, Ross GS, Fernandez HL. Platelets are the primary source of amyloid beta-peptide in human blood. *Biochem Biophys Res Commun* 1995;213:96-103.

Chinn JA, Posso SE, Horbett TA, Ratner BD. Postadsorptive transitions in fibrinogen adsorbed to biomer: changes in baboon platelet adhesion, antibody binding, and sodium dodecyl sulfate elutability. *J Biomed Mater Res* 1991;25:535-55.

Clawson CC, White JG. Platelet interaction with bacteria. II. Fate of the bacteria. *Am J Pathol* 1971;65:381-97.

Clemetson KJ. Platelet activation: signal transduction via membrane receptors. *Thromb Haemost* 1995;74:111-6.

Cohen AB, Stevens MD, Miller EJ, Atkinson MA, Mullenbach G. Generation of the neutrophil-activating peptide-2 by cathepsin G and cathepsin G-treated human platelets. *Am J Physiol* 1992;263:L249-56.

Coller BS. Interaction of normal, thrombasthenic, and Bernard-Soulier platelets with immobilized fibrinogen: defective platelet-fibrinogen interaction in thrombasthenia. *Blood* 1980;55:169-78.

Colman RW. Hemostasis and thrombosis: basic principles and clinical practice. ed. JB Lippincott cop. Philadelphia. 1994.

Coyle AJ, Spina D, Page CP. PAF-induced bronchial hyperresponsiveness in the rabbit: contribution of platelets and airway smooth muscle. *Br J Pharmacol* 1990;101:31-8.

Cramer EM, Savidge GF, Vainchenker W, Berndt MC, Pidard D, Caen JP, Masse JM, Breton-Gorius J. Alpha-granule pool of glycoprotein IIb-IIIa in normal and pathologic platelets and megakaryocytes. *Blood* 1990;75:1220-7.

Crawford N. The presence of contractile proteins in platelet microparticles isolated from human and animal platelet-free plasma. *Br J Haematol* 1971;21:53-69.

D'Souza SE, Ginsberg MH, Burke TA, Lam SC, Plow EF. Localization of an Arg-Gly-Asp recognition site within an integrin adhesion receptor. *Science* 1988;242:91-3.

D'Souza SE, Ginsberg MH, Burke TA, Plow EF. The ligand binding site of the platelet integrin receptor GPIIb-IIIa is proximal to the second calcium binding domain of its alpha subunit. *J Biol Chem* 1990;265:3440-6.

Dahlbäck B. Blood coagulation. *Lancet* 2000;355:1627-32.

Dahlgren C, Sundqvist T. Phagocytosis and hydrophobicity: A method of calculating contact angles based on the diameter of sessile drops. *J Immunol Methods* 1981; 40:171-80.

Devine DV. The effects of complement activation on platelets. *Curr Top Microbiol Immunol* 1992;178:101-13.

Diacovo TG, Puri KD, Warnock RA, Springer TA, von Andrian UH. Platelet-mediated lymphocyte delivery to high endothelial venules. *Science* 1996a;273:252-5.

Diacovo TG, Roth SJ, Buccola JM, Bainton DF, Springer TA. Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. *Blood* 1996b;88:146-57.

Diaz-Ricart M, Tandon NN, Gomez-Ortiz G, Carretero M, Escolar G, Ordinas A, Jamieson GA. Antibodies to CD36 (GPIV) inhibit platelet adhesion to subendothelial surfaces under flow conditions. *Arterioscler Thromb Vasc Biol* 1996;16:883-8.

Dunlop LC, Skinner MP, Bendall LJ, Favaloro EJ, Castaldi PA, Gorman JJ, Gamble JR, Vadas MA, Berndt MC. Characterization of GMP-140 (P-selectin) as a circulating plasma protein. *J Exper Med* 1992;175:1147-50.

Edmunds LH, Jr. The Sangreal. *J Thorac Cardiovasc Surg* 1985;90:1-6.

Eichholtz T, Jalink K, Fahrenfort I, Moolenaar WH. The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem J* 1993;291(Pt 3):677-80.

Elwing H, Ivarsson B, Lundstrom I. Complement deposition from human sera on silicon surfaces studied in situ by ellipsometry. The influence of surface wettability. *Eur J Biochem* 1986;156:359-65.

Engstad CS, Gutteberg TJ, Osterud B. Modulation of blood cell activation by four commonly used anticoagulants. *Thromb Haemost* 1997;77:690-6.

Eriksson C, Nygren H. Adhesion receptors of polymorphonuclear granulocytes on titanium in contact with whole blood. *J Lab Clin Med* 2001a;137:56-63.

Eriksson C, Nygren H. Polymorphonuclear leukocytes in coagulating whole blood recognize hydrophilic and hydrophobic titanium surfaces by different adhesion receptors and show different patterns of receptor expression. *J Lab Clin Med* 2001b;137:296-302.

Escolar G, White JG. Changes in glycoprotein expression after platelet activation: differences between in vitro and in vivo studies. *Thromb Haemost* 2000;83:371-86.

Evangelista V, Manarini S, Sideri R, Rotondo S, Martelli N, Piccoli A, Totani L, Piccardoni P, Vestweber D, de Gaetano G, Cerletti C. Platelet/polymorphonuclear leukocyte interaction: P-selectin triggers protein-tyrosine phosphorylation-dependent CD11b/CD18 adhesion: role of PSGL-1 as a signaling molecule. *Blood* 1999;93:876-85.

Evangelista V, Rajtar G, de Gaetano G, White JG, Cerletti C. Platelet activation by fMLP-stimulated polymorphonuclear leukocytes: the activity of cathepsin G is not prevented by antiproteinases. *Blood* 1991;77:2379-88.

Feraudi M, Merkt J, Weicker H. Bioluminescent assay for determination of picomole amounts of AMP, ADP, and ATP in adipose and muscle tissue biopsies. *Int J Sports Med* 1981;2:106-9.

Fitzgerald LA, Phillips DR. Calcium regulation of the platelet membrane glycoprotein IIb-IIIa complex. *J Biol Chem* 1985;260:11366-74.

Fox JE. The platelet cytoskeleton. *Thromb Haemost* 1993;70:884-93.

Frojmovic MM, Kasirer-Friede A, Goldsmith HL, Brown EA. Surface-secreted von Willebrand factor mediates aggregation of ADP-activated platelets at moderate shear stress: facilitated by GPIb but controlled by GPIIb-IIIa. *Thromb Haemost* 1997;77:568-76.

Fujimura K, Phillips DR. Calcium cation regulation of glycoprotein IIb-IIIa complex formation in platelet plasma membranes. *J Biol Chem* 1983;258:10247-52.

Furie B, Furie BC. The molecular basis of platelet and endothelial cell interaction with neutrophils and monocytes: role of P-selectin and the P-selectin ligand, PSGL-1. *Thromb Haemost* 1995;74:224-7.

Furlan M. Von Willebrand factor: molecular size and functional activity. *Ann Hematol* 1996;72:341-8.

Gachet C, Astier A, de la Salle H, de la Salle C, Fridman WH, Cazenave JP, Hanau D, Teillaud JL. Release of Fc gamma RIIa2 by activated platelets and inhibition of anti-CD9-mediated platelet aggregation by recombinant Fc gamma RIIa2. *Blood* 1995;85:698-704.

Gawaz M, Ott I, Reininger AJ, Neumann FJ. Effects of magnesium on platelet aggregation and adhesion. Magnesium modulates surface expression of glycoproteins on platelets in vitro and ex vivo. *Thromb Haemost* 1994;72:912-8.

Gawaz M, Reininger A, Neumann FJ. Platelet function and platelet-leukocyte adhesion in symptomatic coronary heart disease. Effects of intravenous magnesium. *Thromb Res* 1996;83:341-9.

Gemmell CH, Ramirez SM, Yeo EL, Sefton MV. Platelet activation in whole blood by artificial surfaces: identification of platelet-derived microparticles and activated platelet binding to leukocytes as material-induced activation events. *J Lab Clin Med* 1995;125:276-87.

George JN, Nurden AT, Phillips DR. Molecular defects in interactions of platelets with the vessel wall. *New Engl J Med* 1984;311:1084-98.

Gerrard JM, Robinson P. Identification of the molecular species of lysophosphatidic acid produced when platelets are stimulated by thrombin. *Biochim Biophys Acta* 1989;1001:282-5.

Ginsberg MH, Xiaoping D, O'Toole TE, Loftus JC, Plow EF. Platelet integrins. *Thromb Haemost* 1993;70:87-93.

Goad KE, Horne MKr, Gralnick HR. Pentosan-induced thrombocytopenia: support for an immune complex mechanism. *Br J Haematol* 1994;88:803-8.

Greco NJ, Tandon NN, Jones GD, Kornhauser R, Jackson B, Yamamoto N, Tanoue K, Jamieson GA. Contributions of glycoprotein Ib and the seven transmembrane domain receptor to increases in platelet cytoplasmic [Ca2+] induced by alpha-thrombin. *Biochemistry* 1996;35:906-14.

Grunkemeier JM, Tsai WB, Alexander MR, Castner DG, Horbett TA. Platelet adhesion and procoagulant activity induced by contact with radiofrequency glow discharge polymers: roles of adsorbed fibrinogen and vWF. *J Biomed Mater Res* 2000a;51:669-79.

Grunkemeier JM, Tsai WB, McFarland CD, Horbett TA. The effect of adsorbed fibrinogen, fibronectin, von Willebrand factor and vitronectin on the procoagulant state of adherent platelets. *Biomaterials* 2000b;21:2243-52.

Gulino D, Boudignon C, Zhang LY, Concord E, Rabiet MJ, Marguerie G. Ca(2+)-binding properties of the platelet glycoprotein IIb ligand-interacting domain. *J Biol Chem* 1992;267:1001-7.

Haimovich B, Kaneshiki N, Ji P. Protein kinase C regulates tyrosine phosphorylation of pp125FAK in platelets adherent to fibrinogen. *Blood* 1996a;87:152-61.

Haimovich B, Lipfert L, Brugge JS, Shattil SJ. Tyrosine phosphorylation and cytoskeletal reorganization in platelets are triggered by interaction of integrin receptors with their immobilized ligands. *J Biol Chem* 1993;268:15868-77.

Haimovich B, Regan C, DiFazio L, Ginalis E, Ji P, Purohit U, Rowley RB, Bolen J, Greco R. The FcgammaRII receptor triggers pp125FAK phosphorylation in platelets. *J Biol Chem* 1996b;271:16332-7.

Harrison P, Cramer EM. Platelet alpha-granules. *Blood Rev* 1993;7:52-62.

Heemskerk JW, Vuist WM, Feijge MA, Reutelingsperger CP, Lindhout T. Collagen but not fibrinogen surfaces induce bleb formation, exposure of phosphatidylserine, and procoagulant activity of adherent platelets: evidence for regulation by protein tyrosine kinase-dependent Ca2+ responses. *Blood* 1997;90:2615-25.

Holmsen H, Weiss HJ. Secretable storage pools in platelets. *Ann Rev Med* 1979;30:119-34.

Hong J, Andersson J, Ekdahl KN, Elgue G, Axen N, Larsson R, Nilsson B. Titanium is a highly thrombogenic biomaterial: possible implications for osteogenesis. *Thromb Haemost* 1999;82:58-64.

Horbett TA, Counts RB. von Willebrand Factor/Factor VIII adsorption to surfaces from human plasma. *Thromb Res* 1984;36:599-608.

Horne MKd, Chao ES. Heparin binding to resting and activated platelets. *Blood* 1989;74:238-43.

Houdijk WP, de Groot PG, Nievelstein PF, Sakariassen KS, Sixma JJ. Subendothelial proteins and platelet adhesion. von Willebrand factor and fibronectin, not thrombospondin, are involved in platelet adhesion to extracellular matrix of human vascular endothelial cells. *Arteriosclerosis* 1986;6:24-33.

Hsu-Lin S, Berman CL, Furie BC, August D, Furie B. A platelet membrane protein expressed during platelet activation and secretion. Studies using a monoclonal antibody specific for thrombin-activated platelets. *J Biol Chem* 1984;259:9121-6.

Ikeda Y, Handa M, Kamata T, Kawano K, Kawai Y, Watanabe K, Kawakami K, Sakai K, Fukuyama M, Itagaki I, et al. Transmembrane calcium influx associated with von Willebrand factor binding to GP Ib in the initiation of shear-induced platelet aggregation. *Thromb Haemost* 1993;69:496-502.

Ikeda Y, Handa M, Kawano K, Kamata T, Murata M, Araki Y, Anbo H, Kawai Y, Watanabe K, Itagaki I, Sakai K, Ruggeri ZM. The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. *J Clin Invest* 1991;87:1234-40.

Israels SJ, Gerrard JM, Jacques YV, McNicol A, Cham B, Nishibori M, Bainton DF. Platelet dense granule membranes contain both granulophysin and P-selectin (GMP-140). *Blood* 1992;80:143-52.

Iwabuchi K, Nagaoka I, Yamashita T. Modulation of neutrophil adherence to endothelial cells by platelet-derived adherence-inhibiting factor through interactions with selectin molecules. *J Leukoc Biol* 1998;63:500-8.

Jackson CM, Nemerson Y. Blood coagulation. *Annu Rev Biochem* 1980;49:765-811.

Jennings LK, Phillips DR. Purification of glycoproteins IIb and III from human platelet plasma membranes and characterization of a calcium-dependent glycoprotein IIb-III complex. *J Biol Chem* 1982;257:10458-66.

Jones KH, Senft JA. An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. *J Histochem Cytochem* 1985;33:77-9.

Joseph M, Auriault C, Capron A, Vorng H, Viens P. A new function for platelets: IgE-dependent killing of schistosomes. *Nature* 1983;303:810-2.

Jy W, Mao WW, Horstman L, Tao J, Ahn YS. Platelet microparticles bind, activate and aggregate neutrophils in vitro. *Blood Cells Mol Dis* 1995;21:217-31.

Kanagaraja S, Lundstrom I, Nygren H, Tengvall P. Platelet binding and protein adsorption to titanium and gold after short time exposure to heparinized plasma and whole blood. *Biomaterials* 1996;17:2225-32.

Kieffer N, Phillips DR. Platelet membrane glycoproteins: functions in cellular interactions. *Annu Rev Cell Biol* 1990;6:329-57.

Klinger MH. Platelets and inflammation. *Anat Embryol (Berl)* 1997;196:1-11.

Knauer KA, Lichtenstein LM, Adkinson NF, Fish JE. Platelet activation during antigen-induced airway reactions in asthmatic subjects. *N Engl J Med* 1981;304:1404-7.

Kolarovic L, Fournier NC. A comparison of extraction methods for the isolation of phospholipids from biological sources. *Anal Biochem* 1986;156:244-50.

Kunicki TJ, Pidard D, Rosa JP, Nurden AT. The formation of Ca++-dependent complexes of platelet membrane glycoproteins IIb and IIIa in solution as determined by crossed immunoelectrophoresis. *Blood* 1981;58:268-78.

Kuroda K, Ozaki Y, Qi R, Asazuma N, Yatomi Y, Satoh K, Nomura S, Suzuki M, Kume S. Fc gamma II receptor-mediated platelet activation induced by anti-CD9 monoclonal antibody opens Ca2+ channels which are distinct from those associated with Ca2+ store depletion. *J Immunol* 1995;155:4427-36.

Lambrecht LK, Young BR, Stafford RE, Park K, Albrecht RM, Mosher DF, Cooper SL. The influence of preadsorbed canine von Willebrand factor, fibronectin and fibrinogen on ex vivo artificial surface-induced thrombosis. *Thromb Res* 1986;41:99-117.

Larsen E, Celi A, Gilbert GE, Furie BC, Erban JK, Bonfanti R, Wagner DD, Furie B. PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. *Cell* 1989;59:305-12.

Leir SH, Chen SH, Lei HY. Horse gammaglobulin-induced thrombocytopenia in anaphylaxis involving sequestration and activation of platelets. *Clin Exp Allergy* 1995;25:273-80.

Li N, Wallen NH, Ladjevardi M, Hjemdahl P. Effects of serotonin on platelet activation in whole blood. *Blood Coag Fibrinolys* 1997;8:517-23.

Macfarlane RG. An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. *Nature* 1964;202:498-9.

Mannaioni PF, Di Bello MG, Masini E. Platelets and inflammation: role of platelet-derived growth factor, adhesion molecules and histamine. *Inflam Res* 1997;46:4-18.

Marcus AJ. Platelets: their role in hemostasis, thrombosis, and inflammation. In: Inflammation: basic principles and clinical correlates. 3rd edition. Gallin JI, Snyderman R. ed. Lippincott Williams & Wilkins. Philadelphia. 1999:p77-95.

Markovitz JH, Tolbert L, Winders SE. Increased serotonin receptor density and platelet GPIIb/IIIa activation among smokers. *Arterioscler Thromb Vasc Biol* 1999;19:762-6.

Mason RG, Read MS, Brinkhous KM. Effect off fibrinogen concentration on platelet adhesion to glass. *Proc Soc Exp Biol Med* 1971;137:680-2.

McEver RP, Bennett EM, Martin MN. Identification of two structurally and functionally distinct sites on human platelet membrane glycoprotein IIb-IIIa using monoclonal antibodies. *J Biol Chem* 1983;258:5269-75.

McEver RP, Cummings RD. Role of PSGL-1 binding to selectins in leukocyte recruitment. *J Clin Invest* 1997;100:S97-103.

McEver RP, Martin MN. A monoclonal antibody to a membrane glycoprotein binds only to activated platelets. *J Biol Chem* 1984;259:9799-804.

McNicol A, Israels SJ. Platelet dense granules: structure, function and implications for haemostasis. *Thromb Res* 1999;95:1-18.

Metzelaar MJ, Clevers HC. Lysosomal membrane glycoproteins in platelets. *Thromb Haemost* 1992;68:378-82.

Michelson AD, Barnard MR, Hechtman HB, MacGregor H, Connolly RJ, Loscalzo J, Valeri CR. In vivo tracking of platelets: circulating degranulated platelets rapidly lose surface P-selectin but continue to circulate and function. *Proc Natl Acad Sci USA* 1996;93:11877-82.

Minamoto Y, Hato T, Nakatani S, Fujita S. Detection of platelet adhesion/aggregation to immobilized ligands on microbeads by an aggregometer. *Thromb Haemost* 1996;76:1072-9.

Minckes O, Freyssinet JM, Rouault C, Pavirani A, Rasmussen U, Boyer Neumann C, Meyer D, Girma JP. Interaction of von Willebrand factor with platelets activated by thrombin or a synthetic 7-amino acid peptide derived from the cleaved thrombin receptor. *C R Acad Sci III* 1994;317:363-70.

Modderman PW, Huisman HG, van Mourik JA, von dem Borne AE. A monoclonal antibody to the human platelet glycoprotein IIb/IIIa complex induces platelet activation. *Thromb Haemost* 1988;60:68-74.

Moolenaar WH. LPA: a novel lipid mediator with diverse biological actions. *Trends Cell Biol* 1994;4:213-9.

Morimoto T, Ogihara S. ATP is required in platelet serotonin exocytosis for protein phosphorylation and priming of secretory vesicles docked on the plasma membrane. *J Cell Sci* 1996;109:113-8.

Narayanan S. Multifunctional roles of thrombin. *Ann Clin Lab Sci* 1999;29:275-80.

Niewiarowski S, Walz DA, James P, Rucinski B, Kueppers F. Identification and separation of secreted platelet proteins by isoelectric focusing. Evidence that low-affinity platelet factor 4 is converted to beta-thromboglobulin by limited proteolysis. *Blood* 1980;55:453-6.

Nydahl S, Frebelius S, Swedenborg J. Thrombin inactivation and the effects of antithrombin and heparin in a recirculating Langendorff preparation. *Thromb Res* 1992;65:365-76.

Nygren H. Initial reactions of whole blood with hydrophilic and hydrophobic titanium surfaces. *Colloids and Surfaces B: Biointerfaces* 1996;6:329-33.

Nygren H, Alaeddin S, Lundstrom I, Magnusson KE. Effect of surface wettability on protein adsorption and lateral diffusion. Analysis of data and a statistical model. *Biophys Chem* 1994;49:263-72.

Nygren H, Elam JH, Stenberg M. Adsorption of coagulation proteins and adhesion and activation of platelets at the blood-solid interface. An experimental study of human whole blood. *Acta Physiol Scand* 1988;133:573-7.

Nygren H, Eriksson C, Lausmaa J. Adhesion and activation of platelets and polymorphonuclear granulocyte cells at TiO2 surfaces. *J Lab Clin Med* 1997;129:35-46.

Nygren H, Stenberg M, Karlsson C. Kinetics supramolecular structure and equilibrium properties of fibrinogen adsorption at liquid-solid interfaces. *J Biomed Mater Res* 1992;26:77-91.

Osterman DG, Griffin GL, Senior RM, Kaiser ET, Deuel TF. The carboxyl-terminal tridecapeptide of platelet factor 4 is a potent chemotactic agent for monocytes. *Biochem Biophys Res Commun* 1982;107:130-5.

Packham MA, Evans G, Glynn MF, Mustard JF. The effect of plasma proteins on the interaction of platelets with glass surfaces. *J Lab Clin Med* 1969;73:686-97.

Page CP. Platelets as inflammatory cells. *Immunopharmacology* 1989;17:51-9.

Pasquet JM, Toti F, Nurden AT, Dachary-Prigent J. Procoagulant activity and active calpain in platelet-derived microparticles. *Thromb Res* 1996;82:509-22.

Peerschke EI, Ghebrehiwet B. Human blood platelets possess specific binding sites for C1q. *J Immunol* 1987;138:1537-41.

Peerschke EI, Ghebrehiwet B. Platelet C1q receptor interactions with collagen- and C1q-coated surfaces. *J Immunol* 1990;145:2984-8.

Peerschke EI, Ghebrehiwet B. C1q augments platelet activation in response to aggregated Ig. *J Immunol* 1997;159:5594-8.

Peerschke EI, Reid KB, Ghebrehiwet B. Platelet activation by C1q results in the induction of alpha IIb/beta 3 integrins (GPIIb-IIIa) and the expression of P-selectin and procoagulant activity. *J Exp Med* 1993;178:579-87.

Pineo GF, Hull RD. Thrombin inhibitors as anticoagulant agents. *Curr Opin Hematol* 1999;6:298-303.

Polasek J. Lysosomal concept of platelet secretion–revisited. *Eur J Haematol Suppl* 1989;43:3-24.

Pollard TD, Fujiwara K, Handin R, Weiss G. Contractile proteins in platelet activation and contraction. *Ann NY Acad Sci* 1977;283:218-36.

Pulfer SK, Ott D, Smith DJ. Incorporation of nitric oxide-releasing crosslinked polyethyleneimine microspheres into vascular grafts. *J Biomed Mater Res* 1997;37:182-9.

Ravn HB, Vissinger H, Kristensen SD, Wennmalm A, Thygesen K, Husted SE. Magnesium inhibits platelet activity–an infusion study in healthy volunteers. *Thromb Haemost* 1996;75:939-44.

Rosenfeld SI, Looney RJ, Leddy JP, Phipps DC, Abraham GN, Anderson CL. Human platelet Fc receptor for immunoglobulin G. Identification as a 40,000-molecular-weight membrane protein shared by monocytes. *J Clin Invest* 1985;76:2317-22.

Rotman B, Papermaster BW. Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. *Proc Natl Acad Sci USA* 1966;55:134-41.

Ruf A, Patscheke H. Platelet-induced neutrophil activation: platelet-expressed fibrinogen induces the oxidative burst in neutrophils by an interaction with CD11C/CD18. *Br J Haematol* 1995;90:791-6.

Ruggeri ZM. New insights into the mechanisms of platelet adhesion and aggregation. *Semin Hematol* 1994;31:229-39.

Sako D, Chang XJ, Barone KM, Vachino G, White HM, Shaw G, Veldman GM, Bean KM, Ahern TJ, Furie B, Cumming DA, Larsen GR. Expression cloning of a functional glycoprotein ligand for P-selectin. *Cell* 1993;75:1179-86.

Salvemini D, de Nucci G, Gryglewski RJ, Vane JR. Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor. *Proc Natl Acad Sci USA* 1989;86:6328-32.

Savage B, Ruggeri ZM. Selective recognition of adhesive sites in surface-bound fibrinogen by glycoprotein IIb-IIIa on nonactivated platelets. *J Biol Chem* 1991;266:11227-33.

Savage B, Shattil SJ, Ruggeri ZM. Modulation of platelet function through adhesion receptors. A dual role for glycoprotein IIb-IIIa (integrin alpha IIb beta 3) mediated by fibrinogen and glycoprotein Ib-von Willebrand factor. *J Biol Chem* 1992;267:11300-6.

Sevush S, Jy W, Horstman LL, Mao WW, Kolodny L, Ahn YS. Platelet activation in Alzheimer disease. *Arch Neurol* 1998;55:530-6.

Shattil SJ, Hoxie JA, Cunningham M, Brass LF. Changes in the platelet membrane glycoprotein IIb-IIIa complex during platelet activation. *J Biol Chem* 1985;260:11107-14.

Siess W, Zangl KJ, Essler M, Bauer M, Brandl R, Corrinth C, Bittman R, Tigyi G, Aepfelbacher M. Lysophosphatidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions. *Proc Natl Acad Sci USA* 1999;96:6931-6.

Siljander P, Carpen O, Lassila R. Platelet-derived microparticles associate with fibrin during thrombosis. *Blood* 1996;87:4651-63.

Sims PJ, Wiedmer T, Esmon CT, Weiss HJ, Shattil SJ. Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome: an isolated defect in platelet procoagulant activity. *J Biol Chem* 1989;264:17049-57.

Slupsky JR, Kamiguti AS, Rhodes NP, Cawley JC, Shaw AR, Zuzel M. The platelet antigens CD9, CD42 and integrin alpha IIb beta IIIa can be topographically associated and transduce functionally similar signals. *Eur J Biochem* 1997;244:168-75.

Sobel M, Bird KE, Tyler Cross R, Marques D, Toma N, Conrad HE, Harris RB. Heparins designed to specifically inhibit platelet interactions with von Willebrand factor. *Circulation* 1996;93:992-9.

Stanford MF, Munoz PC, Vroman L. Platelets adhere where flow has left fibrinogen on glass. *Ann NY Acad Sci* 1983;416:504-12.

Stel HV, Sakariassen KS, de Groot PG, van Mourik JA, Sixma JJ. Von Willebrand factor in the vessel wall mediates platelet adherence. *Blood* 1985;65:85-90.

Stenberg PE, McEver RP, Shuman MA, Jacques YV, Bainton DF. A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. *J Cell Biol* 1985;101:880-6.

Stenberg M, Nygren H. The use of the isoscope ellipsometer in the study of adsorbed proteins and biospecific binding reactions. *J de Physique/Colloque* 1983;C10:83-6.

Tan P, Luscinskas FW, Homer-Vanniasinkam S. Cellular and molecular mechanisms of inflammation and thrombosis. *Eur J Vasc Endovasc Surg* 1999;17:373-89.

Tans G, Rosing J, Thomassen MC, Heeb MJ, Zwaal RF, Griffin JH. Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived microparticles. *Blood* 1991;77:2641-8.

Tengvall P, Askendal A, Lundstrom I. Complement activation by IgG immobilized on methylated silicon. *J Biomed Mater Res* 1996;31:305-12.

Thiagarajan P, Tait JF. Collagen-induced exposure of anionic phospholipid in platelets and platelet-derived microparticles. *J Biol Chem* 1991;266:24302-7.

Topol EJ, Byzova TV, Plow EF. Platelet GPIIb-IIIa blockers. *Lancet* 1999;353:227-31.

Triplett DA. Coagulation and bleeding disorders: review and update. *Clin Chem* 2000;46:1260-9.

Tsai WB, Horbett TA. The role of fibronectin in platelet adhesion to plasma preadsorbed polystyrene. *J Biomater Sci Polym Ed* 1999;10:163-81.

Unknown. A new blood-corpuscle. *Lancet* 1882;111-2.

Varma KG, Niewiarowski S, Holt JC, Rucinski B, Paul D. Human platelet basic protein. Its relation to low affinity platelet factor 4 and beta-thromboglobulin. *Biochim Biophys Acta* 1982;701:7-11.

Vik DP, Fearon DT. Cellular distribution of complement receptor type 4 (CR4): expression on human platelets. *J Immunol* 1987;138:254-8.

Vinogradova O, Haas T, Plow EF, Qin J. A structural basis for integrin activation by the cytoplasmic tail of the alpha IIb-subunit. *Proc Natl Acad Sci USA* 2000;97:1450-5.

Vroman L, Adams AL. Identification of rapid changes at plasma-solid interfaces. *J Biomed Mater Res* 1969;3:43-67.

Vroman L, Adams AL, Fischer GC, Munoz PC. Interaction of high molecular weight kininogen, factor XII, and fibrinogen in plasma at interfaces. *Blood* 1980;55:156-9.

Walz A, Zwahlen R, Baggiolini M. Formation and biological properties of neutrophil activating peptide 2 (NAP-2). *Adv Exp Med Biol* 1991;305:39-46.

Waples LM, Olorundare OE, Goodman SL, Lai QJ, Albrecht RM. Platelet-polymer interactions: morphologic and intracellular free calcium studies of individual human platelets. *J Biomed Mater Res* 1996;32:65-76.

Warkentin TE. Heparin-induced thrombocytopenia: IgG-mediated platelet activation, platelet microparticle generation, and altered procoagulant/anticoagulant balance in the pathogenesis of thrombosis and venous limb gangrene complicating heparin-induced thrombocytopenia. *Transfus Med Rev* 1996;10:249-58.

Wencel-Drake JD, Plow EF, Kunicki TJ, Woods VL, Keller DM, Ginsberg MH. Localization of internal pools of membrane glycoproteins involved in platelet adhesive responses. *Am J Pathol* 1986;124:324-34.

Wu YP, van Breugel HH, Lankhof H, Wise RJ, Handin RI, de Groot PG, Sixma JJ. Platelet adhesion to multimeric and dimeric von Willebrand factor and to collagen type III preincubated with von Willebrand factor. *Arterioscler Thromb Vasc Biol* 1996;16:611-20.

Yeo EL, Sheppard JA, Feuerstein IA. Role of P-selectin and leukocyte activation in polymorphonuclear cell adhesion to surface adherent activated platelets under physiologic shear conditions (an injury vessel wall model). *Blood* 1994;83:2498-507.

Yuan Y, Kulkarni S, Ulsemer P, Cranmer SL, Yap CL, Nesbitt WS, Harper I, Mistry N, Dopheide SM, Hughan SC, Williamson D, de la Salle C, Salem HH, Lanza F, Jackson SP. The von Willebrand factor – glycoprotein Ib/V/IX interaction induces actin polymerization and cytoskeletal reorganization in rolling platelets and glycoprotein Ib/V/IX-transfected cells. *J Biol Chem* 1999;274:36241-51.

Zalavary S, Grenegard M, Stendahl O, Bengtsson T. Platelets enhance Fc(gamma) receptor-mediated phagocytosis and respiratory burst in neutrophils: the role of purinergic modulation and actin polymerization. *J Leukoc Biol* 1996;60:58-68.

Zhou D, Luini W, Bernasconi S, Diomede L, Salmona M, Mantovani A, Sozzani S. Phosphatidic acid and lysophosphatidic acid induce haptotactic migration of human monocytes. *J Biol Chem* 1995;270:25549-56.

Zucker MB, Vroman L. Platelet adhesion induced by fibrinogen adsorbed onto glass. *Proc Soc Exp Biol Med* 1969;131:318-20.

GÖTEBORGS UNIVERSITET
GÖTEBORGS UNIVERSITETSBIBLIOTEK

Digitaltryckt & Bunden
Vasastadens Bokbinderi AB
Göteborg 2001