Dynamics of von Willebrand Factor-Mediated Platelet Aggregation in Laminar Flow: Physical and Molecular Determinants

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RATIONALE AND OBJECTIVES

The recruitment of platelets into aggregates is central to both hemostasis and thrombosis, with adhesive ligands such as von Willebrand factor and fibrinogen implicated in both processes. The role of the important adhesive ligand von Willebrand factor in platelet aggregation, has previously been studied mostly at pathologically high shear rates in the absence of chemical modulators, or using maximally activated platelets at more physiological shear rates. However, different agonists and concentrations are expected to variably drive platelet activation responses thereby modifying availability of surface expressed secreted proteins as well as receptor numbers and conformations for platelet aggregation. Therefore, in this study, we examined the role of von Willebrand factor in mediating shear-associated aggregation of platelets at physiologically relevant shear rates and under varying agonist conditions representative of normal physiology or pathology. The participation of additional adhesive ligands is also addressed.

ABSTRACT

Von Willebrand factor (vWF) is a large multimeric protein found in plasma, intracellular stores of platelets and endothelial cells, as well as in the extracellular matrix. VWF has been implicated in venous and arterial thrombosis. Its importance in the primary adhesion of platelets at sites of vascular injury, and for platelet aggregation at very high shear rates was clearly demonstrated by other investigators. We have investigated the role of vWF in the recruitment of platelets activated with low concentrations of agonist, such as may be found with partial ADP secretion or thrombin generation in vivo, at physiologic shear rates (G) ranging from 100 - 2000 s^{-1}.

In the presence of ristocetin, soluble vWF bound to the glycoprotein Ib receptor (GPIb), and mediated shear-associated aggregation independently of the glycoprotein IIb-IIIa receptor (GPIIb-IIIa), with very few vWF monomer equivalents required to achieve high capture efficiencies (α_G; reflecting initial rates of aggregation). Activation of washed platelets in the absence of soluble ligands, with low concentrations of the physiologic agonists, ADP or thrombin, resulted in good aggregation. Participation by GPIb was shear-rate dependent, with the extent of contribution further varying with activation conditions. Inhibition of vWF-GPIb interactions in ADP and thrombin-induced aggregation, caused 25 and 50 % inhibition of α_G at G = 300 and 1000 s^{-1} respectively. α_G's were similar for both agonists, and showed an absolute dependence on activated GPIIb-IIIa in each case. Further investigations using thrombin versus ADP as activator, however, revealed differences in participation by other surface-expressed ligands, in particular Fg. Thus, antibodies against

TSP and Fg inhibited aggregation differentially, depending on shear rate and agonist activating conditions. Removal of catalytically-active thrombin from its receptors by antagonists hirudin and PPACK at ≥ 1 minute following activation, allowed normal secretion to occur from α-granules (monitored using P-selectin). However, the thrombin antagonists significantly decreased both platelet aggregation and surface-expression of vWF and TSP for platelets activated at low (0.05 U/ml), but not intermediate (0.2 U/ml) thrombin concentrations. In conclusion, all our studies demonstrated a consistently important cross-bridging role for vWF, but multi-ligand, multi-receptor participation was required for optimal shear-associated aggregation of platelet suspensions activated at very low agonist concentrations.

RESUME

Le facteur von Willebrand (vWF) est une protéine multimérique présente dans le plasma et la matrice extracellulaire, et stoquée par les plaquettes et les cellules endothéliales. Il est impliqué dans les thromboses veineuses et artérielles. Son rôle majeur dans l'adhésion primaire des plaquettes aux sites de lésions vasculaires, ainsi que dans l'agrégation des plaquettes à taux de cisaillement très élevés a été clairement démontré. Nous nous sommes intéressés à son rôle dans le recrutement des plaquettes faiblement activées, comme c'est le cas *in vivo*, avant la libération (ADP) ou la génération (thrombine) maximale d'agonistes, à des taux de cisaillement physiologiques ($G = 100\text{-}2000\ s^{-1}$) .

En présence de ristocétine, le vWf soluble se lie à la glycoprotéine Ib (GPIb) et supporte l'agrégation des plaquettes en flux, indépendamment de la glycoprotéine IIb-IIIa (GPIIb-IIIa). A peine quelques molécules de vWf sont nécessaire pour atteindre une efficacité de capture (α_G) élevée (α_G reflète le taux initial d'agrégation). En l'absence de ligand soluble, de faibles concentrations d'agonistes physiologiques (ADP ou thrombine) induisent une bonne agrégation des plaquettes lavées. La participation de GPIb est dépendante du taux de cisaillement et des conditions d'activation. Lorsque l'agrégation est induite par l'ADP ou la thrombine, l'inhibition de l'axe vWf-GPIb entraine une baisse de α_G de 25 et 50% pour des taux de cisaillement de 300 et 1000 s^{-1} respectivement. Les α_G sont similaires pour les deux agonistes, et la GPIIb-IIIa activée est indispensable dans chaque cas. Des études supplémentaires en utilisant la thrombine versus l'ADP, ont cependant démontré des différences dans la participation d'autres ligands exprimés à la surface des plaquettes, en

particulier le fibrinogène. L'utilisation d'antagonistes comme l'hirudine et le PPACK pour décrocher la thrombine catalytiquement active de son récepteur, à ≥ 1 minute d'activation, n'inhibe pas la sécrétion normale des granules "α"(verifiée par la détection de P-séléctine à la surface des plaquettes). Cependant, ils entrainent une baisse significative de l'agrégation plaquettaire, ainsi qu'une diminution de l'expression du vWf et de la TSP à la surface des plaquettes, à des concentrations basses (0.05 U/ml), mais pas intermédiaires (0.2 U/ml) de thrombine. En conclusion, dans toutes nos études, nous avons trouvé un rôle important pour le vWf, mais la participation de plusieurs ligands et récepteurs était requise pour optimiser l'agrégation de plaquettes en suspension, à des concentrations très basses d'agonistes.

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It is difficult for me to decide whom to thank first: my ever-supportive family or my supervisor. Without my family's help and understanding, I would not have had the opportunity to complete these studies.

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PREFACE

I have chosen to write this thesis in the manuscript style. As such, I have reproduced the following paragraphs, as required by the Faculty of Graduate Studies and Research for thesis preparation.

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral

defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all authors of the co-authored papers.

Chapter 2. This work has been published: "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. My contribution to this work was consisted of a series of experiments examining the role of glycoprotein receptors GPIb and GPIIb-IIIa under uniform laminar shear flow in the microcouette. I also contributed to discussions pertaining to the work, and which were incorporated into the manuscript. Drs. Frojmovic (my supervisor) and Goldsmith directed the research, while Elizabeth Brown was the technician for Dr. Frojmovic's lab.

Chapter 3. This work has been published: "Kasirer-Friede A, Frojmovic M.M.. Ristocetin- and thrombin-induced platelet aggregation at physiological shear rates: differential roles for GPIb and GPIIb-IIIa receptor." Thromb Haemost 1998; 80: 428-36. I did all the experimental work for this paper; Dr. Frojmovic supervised the research.

Chapter 4. This work has been published: "Kasirer-Friede A, Legrand C, Frojmovic MM: Thrombin receptor occupancy modulates aggregation efficiency and platelet surface expression of von Willebrand factor and thrombospondin, at low thrombin concentrations." Thromb Haemost, 1999; 81: 967-75. I did all the experimental work. Dr. Frojmovic supervised the research, while Dr. Legrand is a collaborator from Unité 353 INSERM, in Paris, France.

Chapter 5. This work has been submitted for publication: " Kasirer-Friede A, Legrand C, Frojmovic MM. Complementary Roles for Fibrinogen, Thrombospondin and von Willebrand Factor in Mediating Aggregation of Platelets Stimulated at Threshold Thrombin Concentrations, in Flow". Submitted to Arteriosclerosis Thrombosis and Vascular Biology, May 1999. I did all the experimental work. Dr. Frojmovic supervised the research, while Dr. Legrand is a collaborator from Unité 353 INSERM, Paris,France.

The references are numbered in the order of their appearance, and are listed separately in each chapter.

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Chapter 1

LITERATURE REVIEW

1. HEMOSTASIS AND THROMBOSIS

Blood circulates within, but under normal circumstances does not interact with, a high-pressure complex network of vessels lined with intact endothelium. It is comprised of three types of blood cells, present at different concentrations: red blood cells (RBC) at 4-6 x $10^6/\mu l$, leukocytes (WBC) at 4-10 x $10^3/\mu l$, and platelets (2-4 x $10^5/\mu l$), suspended in a fluid called plasma. Plasma is composed of approximately 93 % water, various salts, fats, and proteins including those involved in mediating cell-wall (adhesion) and cell-cell (aggregation) interactions, as well as unactivated coagulation factors of the hemostatic clotting cascade. Upon vascular or cellular damage, there is vasoconstriction of vessels, platelet plug formation, and the sequential activation of a series of coagulation factors culminating in the formation of thrombin, which cleaves fibrinogen (Fg) to yield a meshwork of insoluble protein, fibrin.

The regulation of the activation state of cellular receptors and hemostatic precursors allowing participation in a hemostatic mechanism which is "hair-spring"-triggered, requires an exquisitely balanced system to safe-guard against accidental release of pro-coagulant cascades (cellular and chemical), but to nevertheless provide adequate protection against hemorrhage, upon challenge by vascular injury. Platelets play a crucial role in thrombus formation in normal hemostasis (arrest of bleeding) and in pathology (thrombosis). Micro-environmental variations in physical determinants (flow regime) and soluble chemical mediators may modify numbers and sizes of aggregates. Thus, upon vascular injury, exposure of subendothelial layers, rich in collagen and in adhesive proteins von Willebrand factor (vWF) and thrombospondin (TSP) initiate the adhesion of a primary layer of platelets

to subendothelial matrix (Fig. 1-1), highly dependent upon vWF and platelet GPIb. Platelets then become activated [1], locally releasing granule contents including agonists (ADP) and adhesive ligands (VWF, Fg and TSP) [2]. Negatively-charged phospholipids become exposed on platelet surfaces, and catalyze an explosive production of thrombin for further recruitment of platelets into aggregates and for fibrin generation [3].

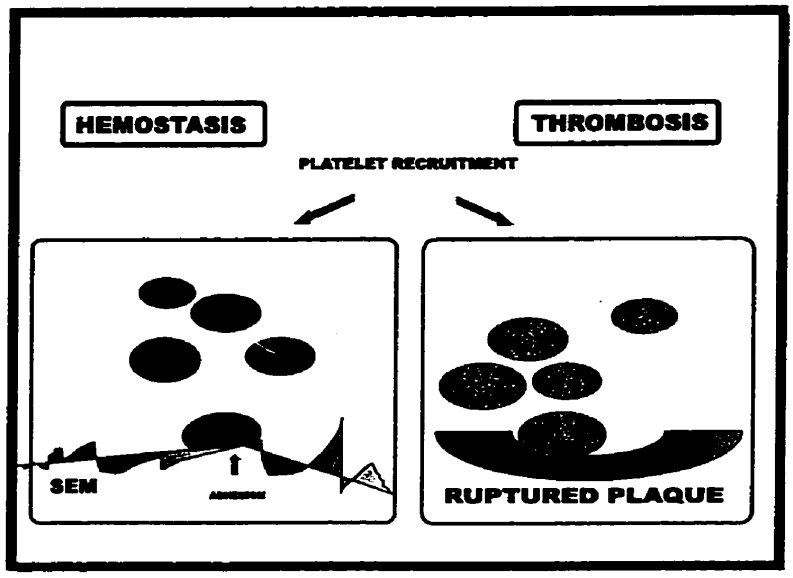

Fig. 1-1. Platelet aggregation is central to both hemostasis and thrombosis. Platelet plug formation at sites of vascular injury may be initiated by exposure of subendothelial matrix (SEM), while the remaining atheromatous material following breakage and removal of the fibrous cap of an atherosclerotic plaque, can also promote thrombus formation.

Thrombus formation at undesired locations in the arterial tree can lead to angina and myocardial infarction, with cardiovascular disease being one of the major causes of death in

developed countries. In arterial thrombosis, plaques develop slowly over a period of years, with cyclic episodes of plaque rupture and rapid platelet aggregation (white thrombus)[4], as well as intra-thrombus thrombin generation[5]. Thrombi in the venous circulation, while consisting primarily of red blood cells and fibrin (red thrombus), can lead to the entrapment of platelets as well[6]. VWF and Fg have been implicated in both venous and arterial thrombosis[7,8] , while TSP levels are elevated in the thickened intima in human vascular disease[9].

2. VASCULAR WALL [10]

Vessel walls are generally composed of three layers, or tunicae, and are called the intima, the media and the adventitia (TH). The innermost layer is the intima and consists of a continuous endothelial cell monolayer and the subendothelial connective tissue, and is bounded by the internal elastic lamina. The subendothelial layer may contain collagens, elastin, fibronectin, laminin, microfibrils, proteoglycans, TSP, VN, and vWF, with the specific proteins expressed varying with the vascular bed[11]. The major cell type is the smooth muscle cell. This layer is important in atherosclerosis since lipid accumulation is targeted primarily to this vessel layer. The tunica media consists of layers of smooth muscle cells, and is separated from the adventitia by the external elastic lamina. The adventitia is made up of fibroblasts, small vessels and nerves, and connects the vessel wall to the surrounding connective tissue sheath. Endothelial cells serve as a selective semi-permeable barrier between tissues and blood. There are about 10^{13} cells which line 7 m^2 of vessel surface[12], with the greatest surface area attributable to capillaries.

There are several self-protective mechanisms which can downregulate pro-coagulant processes. Endothelial cells (EC) produce the potent vasodilators nitric oxide (NO =EDRF) and prostacyclin, which can both inhibit platelet activation, as well as components of the fibrinolytic system[11]. Anti-thrombin III in combination with heparan sulfate, found along vessel walls, can inactivate locally formed thrombin[13]. Endothelial cells may however be pro-coagulant when activated. Thus, upregulation of endothelial selecting and cellular adhesion molecules may promote the adhesion of platelets and leukocytes.

2. 1. Collagen [14]

The collagens are a physiologically important group of proteins which can serve as a template for platelet adhesion and as platelet agonists. They can be subdivided into fibrillar and nonfibrillar structures, and can be further subdivided into several subtypes. The basic structural unit of a collagen fiber (tropocollagen) is that of a heterotrimer composed of two identical chains termed $\alpha 1(I)$ and a third chain called $\alpha 2(I)$. Individual chains are called α-chains, and are numbered in arabic numerals. These are organized into a triple helical conformation (important for function), and intertwine to form a coiled-coil[15]. All alpha chains have repeating Gly-X-Y sequences, but differ in the exact amino acids and chain length. There are nineteen different types of collagen. Collagens Types I, II ,III, V, XI form fibrils[16], with Types I, III and V being the most prevalent of vessel wall collagens. Types IV, VIII and X form sheets[17], and there are other heterogenous groups. The ratio of collagen types in normal versus atherosclerotic vessel wall is different[18]. Collagen type may additionally be correlated with subcellular localization[14], thereby modulating reactivity depending on severity of injury. Interstitial, or fibrillar collagens Types I and III are found

in the medial layers of the aorta and are characterized by continuous and minimally interrupted triple helices. They form insoluble fibers with high tensile strength. There are non-collagenous parts of fibrillar collagen molecules with high homology to adhesive proteins like TSP [19]. Collagen Type VI forms microfibrills[20,21]. It is found in the subendothelium immediately beneath the EC layer, and co-localized with vWF [22]. Collagen Type VI has a short triple helical domain, with very large amino and carboxyl-terminus domains, and has been modeled as a flexible anchor between collagen fibers [23]. There are 11 RGD sequences and several repeats with homology to vWF A domain and to type III repeats of fibronectin (FN)[19].

2.1.1. Platelet-Collagen Interactions

In vitro, platelets adhere to collagen coated surfaces, and form aggregates. Alternatively, collagen may induce platelet aggregation in a stirred suspension, with processes required for adhesion versus activation of platelets being distinct. Several lines of evidence have suggested that there is more than one receptor for collagen on platelets, with the candidates being: $\alpha_2\beta_1$ (GPIa/GPIIa; VLA2), GPIV (CD 36), and GP VI. Platelet adhesion to collagens types I, III, IV and VI, using the blood of a patient lacking the α_2 subunit, is greatly reduced compared to adhesion from normal patients' blood [24]. Similarly, the complete inhibition of platelet adhesion to collagens Types I-VIII caused by a monoclonal antibody directed against the α_2 subunit, under flow conditions, indicates that $a_2\beta_1$ is the principal integrin required for adhesion[25]. This adhesion is Mg^{2+} dependent[26]. GPIV, although first thought to be important, was later shown to be of minor consequence[27]. GP VI, although not required for platelet

adhesion, appears to be necessary for the second, activation phase, and essential for subsequent platelet aggregation[28]. The adhesive molecules FN and vWF may also mediate collagen platelet interactions, by acting as bridging molecules, whereby they may attach to collagen in the vessel wall, as well as to their respective platelet receptors[29].

There are several studies which have been conducted with synthetic peptides in order to identify highly specific reactive sequences. However, it was determined that structural considerations were most important. Collagen-related peptides (CRP's), composed simply of a repeat Gly-Pro-Hyp sequence (Hyp = hydroxyproline; characteristic of collagens and occurring with low frequency in other proteins), can spontaneously assume a triple helical structure. These peptides can "polymerize" in solution when cross-linked by gluteraldehyde, and are highly reactive[30]. By association with GPVI, they may mediate platelet adhesion and activation in a Mg^{2+}-independent manner, determined by observation of increases in intracellular calcium, and phosphotidyl serine exposure on platelet surfaces[26].

2.1.2. Platelet Collagen Interactions under Flow:

Adhesion of platelets can occur on either monomeric or fibrillar collagen under flow[31]. However, fibrillar (polymerized) collagen is more effective than the monomeric form for inducing platelet aggregation [26]. Collagen-related peptides, can support platelet adhesion under static conditions on monomeric or cross-linked (polymeric; CRP-XL) CRP's , but are unable to support platelet adhesion under flow to CRP's in either form, suggesting that additional epitopes are required for providing resistance to shear stress[31]. Platelet adhesion to fibrillar collagen increases with shear rate, up to $G \geq 3000\ s^{-1}$ [32], and requires vWF and platelet GPIb. Adhesion to collagen Type VI has been reported to occur at shear rates $\leq G =$

300 s^{-1} [25], although there is one report suggesting that this interaction can also occur at G as high as 2000 s^{-1} [33]. The ability of monomeric CRP's to minimally activate platelets in solution[34], yet nevertheless antagonize platelet activation induced by CRP-XL or collagen fibers, and to support platelet adhesion under static conditions when immobilized, suggests that multivalency may be an additional requirement for platelet activation by CRP's.

3. SHEAR & FLOW [35,36]

3.1. Shear and blood circulation

Blood flowing through the vessels can be modeled by Poiseuille flow. Due to frictional forces between the vessel wall and the layer of blood immediately adjacent to the wall, there is a no slip condition at the boundary between the two (i.e. velocity = 0). Consequently, there is a series of laminar fluid layers travelling with a parabolic gradient of fluid velocities, generating a shearing stress, with fluid at the center of the tube travelling with maximal velocity. The shear rates (G) will then vary with radial distance from the tube center (r), with maximal shear rates occurring at the vessel wall, such that: $G(r) = 4\,Q\,r / \pi\,R^4$, where Q = volume flow rate, and R = tube radius. Table 2-1 shows the variation in average wall shear rate as one proceeds through the vasculature. However, convective transport will convey cellular or chemical material along streamlines parallel to the endothelial wall, and diffusion is required for substances to reach the surface. Hemodynamic pressure gradients drive bulk flow, but concentration gradients drive diffusion. Fluid layers layers in the vicinity of the wall may be enriched with platelets and leukocytes at higher G as a result of red blood cell

collisions enhancing the radial dispersion of platelets and increasing the platelet diffusion coefficient (D). This, together, with the reduced fluid velocities in this layer, likely encourages platelet-endothelial interactions.

Table 1. Mean Wall Shear Rates Encountered in the Human Circulation

VESSEL	MEAN WALL SHEAR RATE (S^{-1})	REFERENCE
Ascending aorta	245 - 870	37
Femoral artery	300	38
Common carotid	200-400	36,39
Small arteries	1335	36
Arterioles	1600	36
Capillaries	265-2000	36
Veins	20-200	40
Stenotic vessels	800 - 10,000	40

Flow is a very important regulator of events within the vasculature. Regional differences in fluid velocity (and hence shear rate) will regulate the kinetics of platelet arrival at the endothelial surface. However, the reactivity of platelet with the wall will depend on changes in the homeostability of endothelium, where de-endothelialization can provide de-novo molecules. Activation of endothelial cells, will up-regulate the affinity or the numbers

of constitutively expressed molecules, upon which the adhesive potential of platelets is greatly enhanced. Nevertheless, bond formation between travelling platelets and stationary endothelial molecules will depend initially on the kinetics of platelet arrival, then on intrinsic biomechanical properties between reactants which determine contact times required for proper molecular orientation for bond formation. Any reactive molecule on a platelet must reach past the layer of fluid molecules which must be displaced between it and the endothelium, and must overcome repulsive forces generated by the negatively charged outer cellular layers. Thus, strategies adapted by cells towards this end, include the concentration of L-selectin (LECAM-1) on tips of microvilli[41], and the formation of pseudopods upon platelet activation. Variations in shear rate will also modify the local kinetics of convective transport, thereby increasing frequencies of collision between cells and vessel wall, or between two cells. Interaction between the two, however, will further depend on their activation state. Convective transport also serves to dilute out locally generated or secreted vasoactive substances.

For a Newtonian fluid, there is a linear relationship between the shear rate and the shear stress (σ) on a particle, such that $\sigma = \eta G$, where η is the fluid viscosity, representing intermolecular cohesion forces (fluid friction) which must be overcome for fluid motion. Blood can only be considered a Newtonian fluid for $G \geq 100\ s^{-1}$, since blood viscosity can greatly increase at very low G[42] due to the formation of linear and branched rouleaux of red cells.

The relative importance of inertial versus frictional forces can be determined by calculating the dimensionless Reynolds number, Re, where $Re = 2\ R\ U\ \rho / \eta$. Thus, in small

vessels such as arterioles, Re numbers are low and viscous forces are most important, whereas in larger vessels where there are higher fluid velocities and Re numbers, inertial effects pre-dominate. At critical Re numbers (approximately 2300), the laminar fluid streamlines break down, and turbulent flow results.

3.2. Consequences of vessel geometry

There are several branch points (bifurcations), or regions of sudden expansion, which occur in the vasculature. For high bifurcation angles, or relatively large changes in the ratio of cross-sectional area of vessels, $\alpha = \pi(R_1^2 + R_2^2)/\pi R_0^2$, (with α in the circulation usually < 1.3), regional flow separations and recirculation zones may occur. For instance, in the internal carotid bifurcation[43], at a sudden expansion, fluid will suddenly decelerate. Although fluid travelling at the center of the tube may have sufficient inertia to continue flowing, fluid near the vessel wall will not have enough energy to overcome the adverse pressure gradient, and will reverse and form re-circulation zones and vortices. Thus, cells will remain trapped in these regions, with single cells or small aggregates eventually reaching a re-attachment point and reentering the circulation. Studies have shown, that although there is minimal cellular adhesion at the re-attachment points themselves[44,45], likely due to decreased delivery of cells, there is maximal adhesion immediately prior to or after this point. There may also be rouleaux formation near separation and re-attachment points due to the lower shear rates in the recirculation zones.

3.3 Pathological Consequences of ShearVariations

There have been correlations shown between vessel regions prone to atherosclerosis and to areas where sudden changes in vessel geometry occur, resulting in low flow regions

at bifurcations or sudden expansions, where secondary flow patterns may occur as described above. Such vessels include the human carotid artery[43], and aortic T-junctions. Thus, it is expected that there is reduced efflux of small amounts of cellular agonists, normally diluted out by flow. The question however remains, as to whether fluid stasis in these areas is the causative agent for atherosclerosis generation or secondary to it. Kornet et al[39] ,have shown that a low wall shear rate near bifurcations may be associated with larger intima-media thickness. Increased vascular permeability may be also be induced by cholesterol oxidation products, resulting in increased cholesterol levels in the aorta, despite plasma levels within the normal range[46]. High shear rates may induce increased permeability as well, through perhaps different mechanisms. Experiments by Fry et al[47], have demonstrated increased endothelial cell swelling and deformation leading to increases in albumin permeability, in areas subjected to high shear stresses. Stein et al[48], have demonstrated a lack of turbulence at the bifurcation of the abdominal aorta and common iliac arteries, using a hot-film velocimeter, suggesting that turbulence was not a primary cause of atherosclerosis generation, but rather that turbulence may increase further thrombus growth. Nevertheless, atherosclerotic regions may bulge into vessels, creating stenoses. Thus there are several consequences: 1) cells passing through stenotic regions are exposed to extremely high shear rates for very brief periods of time, which may activate platelets[49]; 2) there are new re-circulation zones created immediately downstream of the stenoses; and 3) stresses exerted by fluid flowing past a thrombus may cause thrombus fragmentation (embolism), which may cause plugging of a smaller vessel.

3.4. Cellular effects of high shear

At shear stresses between 1 - 10 dynes/cm^2, there are counter-coagulant endothelial cell protective mechanisms which are activated[50]. Endothelial cells can align with the flow at high shear rates, thereby reducing tensile stress on cells. Shear has been shown to activate Ca^{2+}, and K^+ channels in endothelial cells, or integrins, which may act as mechanotransducers, resulting in up regulation of atheroprotective mechanisms, eg. tissue-plasminogen activator (t-PA[51]), nitric oxide (NO[52]), or pro-coagulant adhesive ligand vWF[53]. High shear stresses however, appear to have a pro-coagulant effect on platelets. Brown et al[54] have shown that platelets sheared at G = 5000 s^{-1}, may release some stored ADP, which can locally activate platelets, or platelets may fragment at still higher G. Shear rates upwards of G = 6000 s^{-1} can also induce platelet aggregation[55].

3.5. Experimental investigation of shear effects

Several devices have been used to study the effects of shear on cellular interactions with the vessel wall (adhesion), or with each other (aggregation). Parallel plate perfusion chambers have been developed where either whole blood, or suspensions of platelets or leukocytes suspended in buffer or plasma can be perfused over slides coated with everted aortas, SEM or purified adhesive substances[56]. The rate and extent of adhesion can be determined by microscopy or by morphometric analysis. The narrow chamber width yields a relatively constant laminar wall shear rate for a given rate of perfusion, where G = du/dy, where u = velocity. Other devices used to examine cellular aggregation include the aggregometer, the cone and plate viscometer, the couette (Fig. 1-2) and a tube flow device.

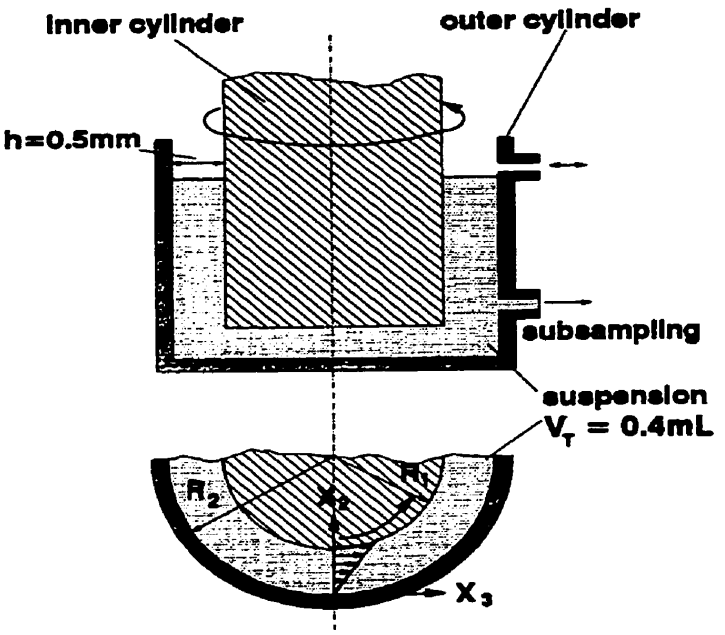

Figure 1-2. Microcouette. A microcouette composed of two concentric plexi-glass cylinders of respective diameters of 10.0 mm (i.d.) and 11.0 mm (o.d.) with a gap width h = 0.5 mm. The inner cylinder, driven by a high precision step motor, rotates at a desired angular velocity, with respect to the stationary outer cylinder, to yield a simple shear flow in the space between the concentric cylinders (from Xia et al, Biophys J 1994; 66: 2190-201, with permission).

Aggregometers coupled to a light transmission detection system were traditionally used to investigate cell aggregation in flow. However, flow dynamics may be turbulent and difficult to define , with shear rates generated estimated to $\leq 100\ s^{-1}$. Both the couette and the cone-and-plate viscometer can generate uniform laminar shear flow, at shear rates in the physiologic and pathologic ranges. Tube flow devices may be used to generate Poiseuille flow, as described previously, with G in the physiologic range. For the experiments presented here, we have used a microcouette device consisting of two concentric cylinders. The outer

cylinder is maintained stationary, while the inner one is rotated at a frequency ω, and $G = \omega r/h$, for $h \ll r$, where r = radius of the inner cylinder and h = the gap width between the cylinders. Experimentally, the percent aggregation (% PA) of homotypic cells in suspension can be estimated by determining the decrease in platelet number with time, i.e. $\% PA = (1 - N_t / N_0) \times 100$ % at a given time point, t, where N_t = number of cells remaining at t, and N_0 is the initial number of cells. A theoretical formulation of the frequency of a particle colliding with a second particle to form a doublet may be derived using Smoluchowski's equations for the collision frequency of a dilute suspension of rigid spheres of radius a, i.e. the two-body collision frequency per unit volume of suspension is given by $(CF) = 16/3\ G\ a^3\ N_0$ [57]. This can be compared to the experimentally determined initial particle aggregation for very early times when only doublets and a few multiplets are formed, and used to calculate the capture efficiency, α_G , such that $\alpha_G = dPA/dt / CF$[58].

4. VON WILLEBRAND FACTOR

4.1. Synthesis and binding epitopes

von Willebrand factor (vWF) is a very large glycoprotein present in plasma at concentrations of 5 - 15 -μg/ml. It circulates as a series of variably lengthed multimers, with molecular weights ranging between 500 and 20,000 kDa[59]. Each multimer consists of repeating subunits of approximately 240,000 Da. VWF is synthesized by megakaryocytes (platelet pre-cursors)[60] and by endothelial cells[61], where there is bipolar secretion either into the subendothelium to associate with microfibrillar structures (collagen Type VI[22]), or into the vessel lumen upon platelet activation[63]. The gene for vWF is on chromosome 12 [64], and

contains 52 exons over 178 kilobases[65]. The mRNA transcript is translated to yield a pre-pro-vWF containing a 22-residue signal peptide, a 741-residue pro peptide (also known as vWF-Ag II), and the mature vWF subunit of 2050 residues[64,66]. The signal peptide is cleaved before entering the Golgi and the pro-vWF dimerizes via disulphide bond formation at the carboxyl terminus end[67]. Further polymerization occurs in the Golgi and requires the presence of the propeptide[68]. The propeptide is later cleaved and vWF:AgII can be found non-covalently associated with the mature protein in platelet alpha (α) granules.

VWF has domain organization, with 4 distinct homology domains (A - D): three A, two B, two C and 4 D domains[65]. VWF is highly glycosylated[69], with about 18.7 % of the molecular weight attributable to carbohydrate, and is cysteine rich. Each vWF subunit contains binding sites for platelet receptors and sub-endothelial matrix proteins (Fig.1-3). There are two binding domains for collagen, with the principal site for binding to collagen Type VI located in the vWF A1 domain[33], while the principal binding site for collagen Type III is located in the vWF A3 domain[70]. There are also binding sites for heparin and sulfated proteoglycans. vWF also functions as a carrier protein for clotting cascade factor VIII, by protecting it from proteolysis by activated Protein C. VWF can bind to two platelet glycoprotein receptors; GPIb and GPIIb-IIIa, depending on activation and dynamic conditions, and will be discussed further in a later section. Much effort has gone into characterizing interaction sites on vWF for GPIb. The binding epitope appears to be composed of a series of discontinuous regions in and about the A1 domain. vWF binding to the GPIIb-IIIa receptor is via an RGD site found in each vWF subunit at residues 1744-46[71].

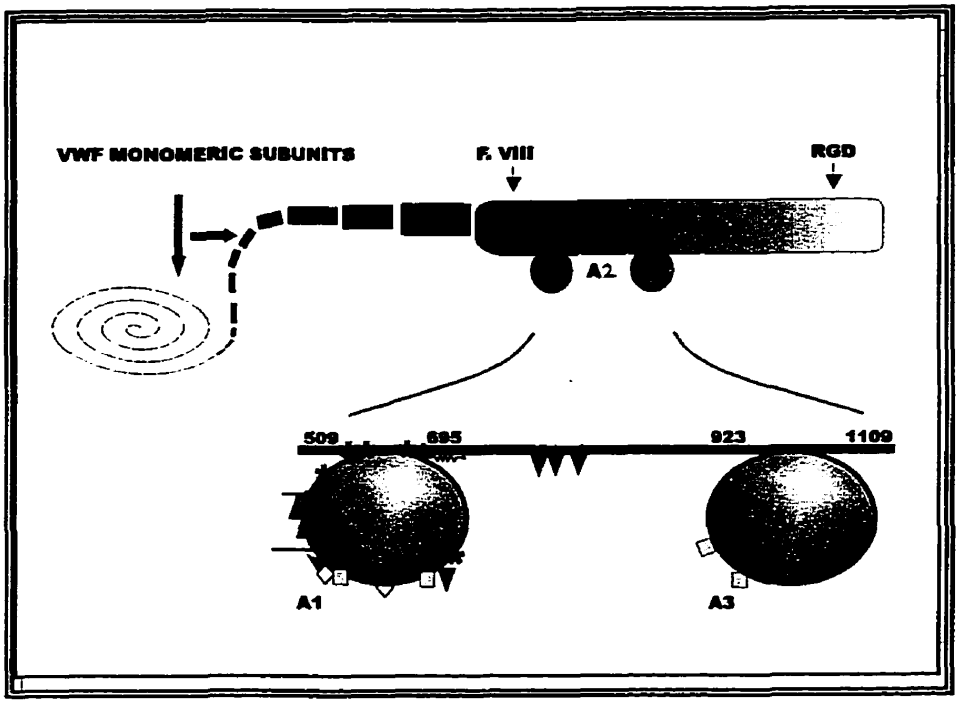

Figure 1-3. Binding sites on von Willebrand factor monomeric subunits. The von Willebrand factor molecule is composed of a varying number of repeating subunits. In solution, it has been shown to exist in a mostly globular state, but it may be extended under conditions of high shear stress. This figure also illustrates the location of epitopes on vWF recognized by extracellular matrix proteins and glycoprotein receptors with which it interacts.

4.2. von Willebrand factor structure

Early studies visualizing the vWF molecule by electron microscopy (EM), show vWF to be an elongated, flexible molecule with small nodules at irregular intervals, with a length of 50-1150 nm and width of approximately 2.5 nm[72]. Large variations in the shape of the vWF molecule have been observed[73]. Studies including data from both electron microscopy

(representing highly processed fixed vWF) and using quasi-electric light scattering (soluble vWF), have suggested that the predominant structure (87%) is a " loosely coiled ball of yarn", with diameter 100-150 nm, and occasionally > 300 nm[73]. This is similar to the patch-like globular appearance of secreted vWF observed on the surfaces of activated platelets[74]. Contour lengths are usually < 500 nm but may occasionally be > 1000 nm. More recently, atomic force microscopy has been used to image vWF in physiological buffer solution adsorbed onto a hydrophobic self-assembled monolayer. The vWF molecule was shown to be globular, with major axis diameter of 106-149 nm, in good agreement with electron microscope studies above, minor axis diameter of 77-81 nm, and height of 3.4-3.8 nM[75,76]. It appeared to be composed of overlapping globular domains, and was thus similar to the "ball of yarn" described in EM studies. Globular domains were further classified as large (L; 66 nm major axis diameter) or small (S; 38 nm), and were shown to alternate as L-S-LL-S-L. The tightly wound configuration of subunits was disrupted to different extents by the application of shear forces of different magnitudes, with medium extension of the molecule due to forces resulting from imaging with the probe tip in contact mode (7-19 pN), and full extension upon application of shear forces of ≥35 dynes/cm^2. Thus, it can be postulated that exposure to such high shear forces may cause physiologically relevant unwinding of the vWF molecule.

Due to the large size of the molecule, X-ray crystallography studies have been conducted only on isolated recombinant A1 or A3 vWF domains. These domains are part of an A (I-domain) homology group including LFA-1, the leukocyte integrin, CR-3 , Type VI collagen[77], and other integrins. The domain organization has also been shown to have

topological similarity to the Ras p-21 protein[78].The solution of the crystal structure has described an A-1 domain with an overall cuboidal shape[79], which is characterized by 5 parallel ß-strands and 1 anti-parallel ß-strand which are centrally located, and flanked on either side by 3 α-helices[79,80]. There are 6 flat faces , including a mostly basic and a mostly acidic one[79]. A salt bridge network runs around the lower rim of the A1 domain. The A1 and A3 domains are not expected to bind metal ions, unlike α_M, α_A, α_2 , since they lack 2-3 residues directly involved in metal ion co-ordination. Molecular homology modeling studies of the A1 domain, suggest that there is a crevice formed close to the point at which ß-strands wind in opposite directions, and is expected to represent an active site[81]. This crevice corresponds to the ristocetin binding site in the A1 domain, and the protease cleavage site in the A2 domain. Celikel et al[80], also using X-ray crystallography, reported pairing of A1 domain molecules close to the crystallographic 2-fold axis of symmetry, with salt bridges occurring between pairs of molecules. This raises the question of how a full-size vWF molecule, shown to be globular, would pack in solution, and whether such salt bridges between A1 domains of neighbouring subunits could contribute towards stabilizing vWF structure.

4.3. vWF in Pathology

4.3.1. von Willebrand Disease

Von Willebrand factor plays an important role in hemostasis, as indicated by a common bleeding disorder first described by Erich von Willebrand in 1926, and named von Willebrand Disease (vWD). Patients afflicted with vWD, inherited mostly as an autosomal

dominant, suffer from mucocutaneous hemorrhaging and epistaxis. VWD affects 5-10 out of 100 individuals[82]. There are three principal types of von Willebrand disease (vWD) with additional subtypes described. These disorders have recently been reclassified, as summarized by Sadler et al[83]. In Type I, the most common form, there is normal multimeric distribution, but reduced vWF plasma concentration. It is difficult to establish genetic defects and only a few have been described. Type II patients have a qualitative defect of vWF function. In Type IIA (loss of function mutations), the large and intermediate multimers are absent, with almost all mutations accounting for this subtype located in the A2 domain. This domain contains a region normally exposed only at higher shear rates[84], and susceptible to cleavage by a newly described plasma protease[85]. There is also a recessive form, which was previously classified as a Type IIC. In Type IIM, also inherited as an autosomal recessive, plasma concentration and multimeric distribution are normal, but there is a qualitative defect in vWF function, with mutations occurring mostly in the A1 domain. Type IIB patients are hyper-responsive to low doses of platelet agonists (gain of function mutation). They also lack high molecular weight multimers, and are thrombocytopenic, presumably due to spontaneous aggregation of the very large multimers[86]. Mutations described for this vWD Type are clustered in the A1 domain. Severe vWD patients, classified as Type III, show virtually no plasma, platelet or endothelial cell vWF. Type III vWD is generally caused by gross chromosomal changes such as deletions, nonsense mutations and frameshift mutations. Type N vWD, or pseudo-hemophilia patients, have mutations in the first 100 amino acids, in the vWF region corresponding to the binding site for factor VIII.

4.3.2. vWF in Thrombosis

Several studies have shown a correlation between plasma vWF concentrations and the incidence of venous and arterial thrombosis[7]. In fact, vWF levels are increased in each of the major risk factors for atherosclerosis, such as diabetes mellitus, and retinopathy[87]. Also, functional changes in vWF may be associated with certain stroke subtypes, where shear-induced platelet aggregation (SIPA) occurs at shear rates below those at which platelets would aggregate without externally-added agonist[88]. In an animal model study, normal and vWD pigs fed an atherogenic diet, were used to investigate the role of vWF in adhesion and thrombus formation in response to mild (angioplasty) and more severe (stenosis and pinch) injury. Decreased vWF levels in vWD pigs led to a decreased response to more severe injuries. Accordingly, occlusive obstruction of the vessel occurred only in normal pigs, suggesting that lowered vWF levels may afford some protection against occlusive thrombus formation[89]. Very few studies have been conducted in humans relating vWD and thrombotic events. Autopsy reports on vWD patients show that although atherosclerotic regions were formed, there were no occlusive thrombi at the time of death, again implicating vWF in acute thrombus growth[90].

4.3.3. vWF in Other Disease States

Unusually large forms of vWF have been described in patients with thrombotic thrombocytopenic purpura (TTP), a condition where microthrombi are spontaneously formed in the circulation, with clearance of aggregates leading to decreased availability of flowing platelets. The aetiology is suspected to be an absence of, or inhibition by an autoantibody against the vWF cleaving protease[85].

4.4. Regulation of vWF-Platelet Interactions:

Von Willebrand factor can interact with platelet glycoprotein receptors GPIb and GPIIb-IIIa, but only under appropriate conditions. Under static or physiologic flow conditions, soluble vWF cannot bind either receptor without mediation by a chemical agonist. The antibiotic ristocetin has been shown to mediate binding to GPIb, where ristocetin dimers may bridge vWF and GPIb by charge neutralization[91]. A venom derived from the Bothrops jararaca snake, botrocetin, can form a complex with vWF (residues449-728 [92]), thereby allowing it to bind to the GPIb receptor. Soluble vWF binding to the activated GPIIb-IIIa receptor can be mediated by the physiologic agonists ADP and thrombin[93]. The physical agonist of shear can independently "activate" vWF and/or GPIb at shear rates $\geq 6000\ s^{-1}$, with initial binding of vWF to GPIb, producing a calcium influx and activation of the GPIIb-IIIa receptor[94].

In contrast to requirements for soluble vWF-GPIb interactions, platelets can directly recognize immobilized vWF via the GPIb receptor. Even at very high shear rates, ($G = 10{,}000\ s^{-1}$), platelets will transiently interact with immobilized vWF, leading to cytoskeletal changes[95] and activation of GPIIb-IIIa[96], required for stable adhesion. Unactivated GPIIb-IIIa does not recognize immobilized vWF, unlike direct recognition of immobilized Fg[97]. vWF has also been shown to associate with thrombospondin in a purified system[98], and to mediate platelet-fibrin interactions[99-101].

Studies with antibodies which selectively inhibit soluble vWF binding to GPIIb-IIIa* [102], suggest that the binding epitope on vWF recognizing GPIIb-IIIa* is comprised of an RGD sequence at residues 1744-1746 on vWF, as well as adjacent residues which may

modify the presentation of the RGD sequence, shown to affect binding affinities. The determination of domains involved in vWF-GPIb binding has been challenging, and is still not completely resolved. Based on recent studies, it would appear that several distinct regions in the vWF molecule contribute to vWF-GPIb binding interactions:1) regulatory domains 2) regions sensitive to allosteric modulation, and 3) epitopes directly constituting contact regions.

There are several lines of evidence which suggest that specific sequences interacting with GPIb are within the A1 domain despite mutations of vWD Type IIA falling mostly within the A2 domain. Studies by Miyata et al[103], using a recombinant A1 domain, in cyclic or in reduced and alkylated form, showed that such domains were able to directly interact with platelet GPIb when in a molten globule state (partly denatured), but not in a native conformation. Similarly, antibodies against regions of GPIb have been shown to decrease vWF-GPIb binding mediated by ristocetin (residues 702-704)): encompassing regions 694-708, 474-488, 514-542) or botrocetin (514-542). Studies by Piétu et al[104], using recombinant epitope mapping found inhibition of vWF binding to GPIb by monoclonal antibodies against Ser 593-Ser 678, and none by antibodies against Ser523-Gly 588. Scanning alanine mutagenesis[105] of the region composed of residues His 463-Gly 716, showed that antibodies against sequences which disrupt recognition by the antibody NMC-4 (used as an index of A1 domain structural integrity) in the presence of ristocetin, include regions which directly inhibit vWF-GPIb interactions: 497-511, the acidic region 687-698 and the basic region 540-578. They concluded that there were inhibitory domains which were neutralized, or alleviated by ristocetin in order for vWF-GPIb interactions to occur.

Expression of six common mutations occurring in the A1 domain of Type IIB vWD patients by Cooney and Ginsburg[106], resulted in spontaneous binding to platelets, but with no further increase in binding when groups of mutations were co-expressed. They concluded similarly to Matsushita et al that there was a discrete on/off vWF conformation, with Type IIB vWF perpetually in the "on" state. This is partly disputed by Miyata et al[107], who argue that constitutive transient interactions between vWF and GPIb occur in plasma, with high association and dissociation rates representing a protective mechanism. Adsorption of vWF would then merely align and increase surface density of binding epitopes and diminish cellular off-rates, thereby allowing stabilization via integrins. In this model, immobilization does not disclose cryptic sites. Immobilization of vWF on variably-sized latex beads under different dynamic conditions have, however, suggested a dependence of vWF-mediated agglutination of fixed platelets on conditions for adsorption of vWF[108]. Differences encountered between disruption of ristocetin versus botrocetin-mediated binding of vWF to GPIb suggest that there may actually be multiple regulatory mechanisms of vWF-GPIb binding, with perhaps differences in further downstream signaling.

Further insights into distinctions between regulatory and binding domains may be had from a combination of mapping vWD mutations to distinct regions of the molecule and from structural studies. Thus, vWD Type IIA mutations fall mostly within the A2 domain, where positive and negative charges are more evenly distributed on the surface than in the A1 domain. Type IIA mutations mostly correspond to cryptic locations, and may therefore alter molecular folding, exposing the protease cleavage site to proteolytic attack, or affecting processing of vWF. Type IIB mutations are found on the lower surface of the domain, near

the botrocetin binding site[79], with many mutations located at the amino edge of the ß-sheet. Molecular modeling studies[81] show that several of the residues represent replacement of surface-expressed loop residues. Others are mutations of small hydrophobic residues buried in the ß-strand and may disrupt the packing of the local protein structure. Type 2M mutations may most accurately reflect actual vWF binding sites on GPIb, since mutations do not affect plasma levels or multimerization, suggesting normal structure. Nevertheless, downregulation of affinity is produced by mutations occurring on loops, above the plane of the molecule and mostly in regions of high solvent accessibility.

4.5. Compartmentalization of vWF

Specific structural and functional differences between vWF in different compartments have been described. Although vWF can be stored in α-granules within platelets or in Weibel-Palade bodies in endothelial cells, plasma vWF is expected to be endothelial cell derived[109]. Multimerization patterns between stored and plasma vWF are similar except that stored vWF also includes very large multimers[109,110]. Subendothelial vWF is composed of dimers and small multimers[109]. Dissimilar glycosylation of platelet and plasma vWF, with sialic acid and galactose content on plasma vWF almost double that on platelet vWF, may shield plasma vWF to a greater extent, due to differences in charge or effective diameter[110].

Functional differences between platelet and plasma vWF are reflected by differences in the K_d of binding to platelets activated with different agonists. Plasma vWF was shown to have a slightly higher affinity for ristocetin-mediated binding to the GPIb receptor, while platelet vWF showed a 10-fold higher affinity for binding to thrombin-activated platelets

than plasma vWF[110]. Indeed, secreted platelet vWF has been shown to associate primarily with GPIIb-IIIa[111]. The extent of inhibition of thrombin-mediated vWF binding to GPIIb-IIIa by a peptide constituting the last 15 residues of the Fg γ- terminus, or an RGDS peptide, also differed for vWF from the two sources. Thus, the IC_{50} for inhibition by γ-15 was approximately 30-fold lower for platelet than for plasma vWF, with synergistic inhibition by both peptides only observed for platelet vWF[112]. Binding to collagen-activated platelets occurred with similar affinities[110].

Although plasma vWF is required for the initial adhesion of platelets to subendothelial matrix, matrix vWF has also been shown to be functionally relevant[1]. Platelet vWF is required for primary hemostasis as well. Platelet transfusions were shown to further shorten bleeding times in Type III vWD patients, lacking plasma and platelet vWF, who had received cryoprecipitate infusion[113]. Platelet vWF has also been shown to normalize bleeding time post DDAVP treatment in Type I patients[114], and to mediate deposition onto purified collagen at G = 1600 s^{-1}, as determined by comparing platelet deposition using normal or vWD platelets and plasma[115,116].

5. OTHER ADHESIVE LIGANDS

5.1. Fibrinogen[117]

Fibrinogen is a dimeric protein produced by the liver, and secreted into the plasma, where it circulates at a concentration of about 7 μM. It is incorporated into platelet α-granules mostly by glycoprotein receptor IIb- IIIa dependent endocytosis[118], to be secreted

upon platelet activation. Fg is not found in the subendothelial matrix, but may be deposited upon vascular injury[119]. Defective or absent Fg results in a bleeding disorder called afibrinogenemia. An increased risk of thrombosis has been associated with dysfunctional Fg lacking amino acids 9-72, which are normally involved in thrombin and plasminogen binding[120]. Other structural variants exist, with the predominant one occurring as a heterozygous condition, where one gamma chain has a 20-residue extension instead of the normal 408-411 sequence[121,122].

Although there is homology between the three Fg chains, important differences between them include the presence of fibrinopeptide A (FPA) and fibrinopeptide B (FPB) on the amino terminus of chains Aα (1-16) and Bß (1-14), respectively. Upon treatment with thrombin, these peptides are cleaved, leading first to fibrin monomer formation, then protofibril assembly, and subsequent polymerization to yield an insoluble clot. Fibrin is stabilized by cross-linkage by activated F.XIII[123,124]. In the course of fibrin formation, new epitopes become exposed, eg. for tPA[125]. Degradation of Fg and fibrin is principally due to cleavage by plasmin, which initially removes a 403-residue sequence from Fg, converting Fg to Fragment X. Further cleavage yields fragments Y , D and E. The presence of D-dimers in plasma has been used as an index of thrombin activity.

5.1.1. Fg Structure

Fg is composed of three pairs of disulfide-bonded polypeptide chains α, ß, γ and has a total MW of approximately 340,000 Da[126]. Doolittle et al[127] showed that there is a Cys-Pro-X-X-Cys sequence occurring twice in each of the three chains, with intervening 111-residue sequences of each chain showing similar polar/nonpolar residue alterations. There is a low

frequency of proline occurrence, suggesting a helical backbone structure. Fg chains are intertwined into a three-stranded rope to form a coiled-coil structure of approximately 160 Å, with each chain possessing an α-helical conformation. Electron microscopy shows a trinodular model for Fg, with a length of 475 Å, with two roughly spherical nodules 65 Å in diameter connected by thin threads to a central nodule of 50 Å diameter. The axial ratio is 5:1. This is in agreement with atomic force microscopy studies, where the hydrated Fg length is shown to range between 480 and 650 Å[128,129]. There are three high affinity calcium binding sites on human Fg[130,131]: one in each of the gamma chains γ-303-356, and another in the central domain of the molecule.

5.1.2. Fibrinogen-Platelet and -Neutrophil Interactions:

Although there are three binding sequences per half Fg molecule potentially available for binding to platelet GPIIb-IIIa: Aα 95-97 RGDF, Aα 572-574 RGDS and γ-400-411 (Fig.1-4), only the gamma terminus sequence is required for the initial binding of Fg[132,133]. This sequence has similarly been shown to be essential for soluble-Fg mediated aggregation of platelets and for the adhesion of platelets to surface-immobilized Fg[134,135]. However, in clot retraction, none of these putative sites appear to be involved[136]. Unlike differences between platelet and plasma-derived vWF, soluble intra-granule Fg is equivalent to plasma-derived Fg when compared by gel electrophoresis, by Kd's for binding to activated platelets, by the ability to support platelet aggregation, and by clottability[137]. Fg binding to platelets is a multiphasic process, with irreversible binding increasing with time, as determined by dissociability with excess Fg or with EDTA , although this phenomenon is less obvious for

binding to fixed platelets[138,139]. There are binding domains for thrombospondin at Aα 92-147 and Bß 113-126 [140].

Studies examining the efficiency of platelet-Fg interactions have shown that the optimum shear range for soluble Fg-mediated interactions is between 50 and 600 s^{-1} [58]. Platelet adhesion to immobilized Fg can occur in the absence of platelet activation[97], although adhesion efficiency in flow increases with activation[141]. Immediate arrest of interacting platelets on Fg optimally occurs at uniform laminar shear rates between 50 and 500 s^{-1} [96] . Fg can also mediate platelet-neutrophil interactions. Thus, soluble Fg can bind GPIIb-IIIa* on platelets as well as MAC-1* or p150/95* on neutrophils[142]. It can also mediate monocyte-endothelial cell interactions by bridging MAC-1 on monocytes to ICAM-1 on endothelial cells[143].

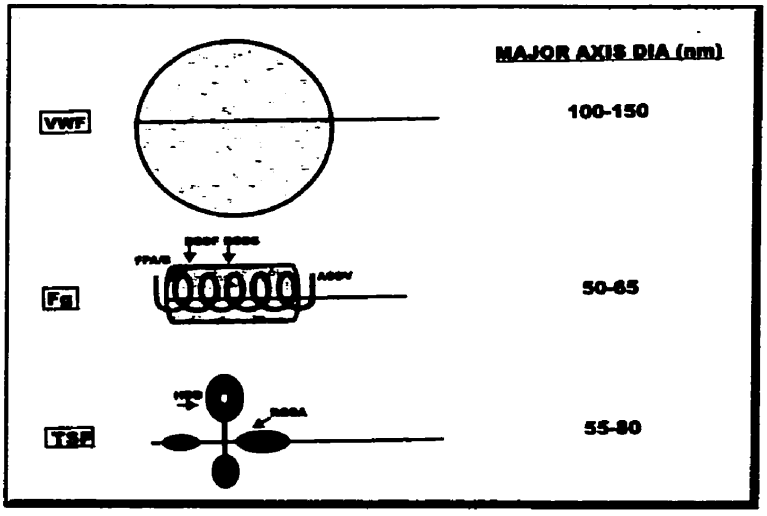

Fig. 1-4. The adhesive ligands vWF, Fg and TSP. Comparison of relative molecular sizes and shapes of the adhesive ligands vWF, Fg and TSP based on rotary shadowing electron microscopy and atomic force microscopy (for vWF and Fg).

5.2. THROMBOSPONDIN [144]

Thrombospondin (TSP) is a large glycoprotein of 450 kDa, which is synthesized by various cells including endothelial cells (EC), smooth muscle cells, fibroblasts, epithelial cells and several tumour cell lines. It is incorporated into the ECM in a vascular bed-specific manner [9]. Although it is found at very low concentrations in plasma (< 200 ng/ml), it is one of the principal proteins (by concentration) stored in platelet alpha granules (~100 ng/ 10^9 platelets).

5.2.1. TSP Structure and binding domains

TSP is composed of three identical chains. Rotary shadowed electron microscopy shows the TSP molecule as having a central globular region connected by thin filamentous regions to three other globular regions[145]. The conformation of TSP is extremely calcium sensitive; there are 12 potential calcium binding sites per chain[146], and its adhesive potential is strongly modulated by calcium availability[147]. Electron microscopy studies have indicated that a doubling in length of the connecting regions can occur in the absence of calcium, as well as a decrease in the diameter of the globular regions. These conformational changes occurring at low calcium concentrations dispose TSP to cleavage by thrombin[148].

There is a heparin binding domain (HBD) at the amino terminal end of the molecule, followed by a series of repeating sequences referred to as the Type I, II and III regions, respectively. Due to the plethora of possible association sites on TSP, there is some controversy regarding binding sites for TSP on platelets, with Fg (with which it can form a stable complex), GPIV, integrin associated protein (IAP), and GPIIb-IIIa proposed. A direct association with vWF has also been shown in solid phase assays[98]. Purified GPIIb-IIIa was

shown to interact with TSP in a solid-phase system[149], likely via an RGDA sequence located in the last Type III repeat. TSP appears to co-localize with Fg and GPIIb-IIIa on activated platelets[150], but requirements for Fg and GPIIb-IIIa have been questioned since there is normal expression of TSP on the surfaces of platelets from patients with severe afibrinogenemia[151]or Glanzmann's Thrombasthenia, where both GPIIb-IIIa and alpha-granule Fg may be absent[152]. Peptides with the sequence RFYVVMWK, corresponding to a region in the carboxyl terminus of TSP, can also bind to platelets via IAP[153], and directly induce platelet aggregation and activation, as defined by phosphorylation of P47 protein [154].

TSP is a multifunctional protein which can participate in both coagulant and fibrinolytic events, wound healing and angiogenesis and can interact with cancer cells[144]. TSP binds matrix proteins heparin, collagen Type V, and fibronectin, and TSP levels are increased in the thickened intima in vascular disease[9]. TSP can also modulate fibrin clot structure, promoting the formation of a finer clot in its presence[155].

5.2.2. TSP modulation of platelet adhesiveness

Peptides derived from the HBD of TSP reduce secretion by up to 50 %, suggesting that TSP can function to amplify platelet signaling processes in ADP and thrombin activated platelets[156]. It is however unclear if this is a direct response, or secondary to inhibition of its interaction with Fg, mediated by the same domain. TSP was previously implicated in maximizing macro-aggregate formation and stabilization, in ADP-activated platelets. Specific domains in the HBD may be involved, with some inhibition of aggregation produced by antibodies against epitopes residing within the Type III repeat regions[157]. Again,

it is unclear whether this is due to disruption of native TSP conformation, or if these are sites directly required for platelet aggregation. Monoclonal antibodies against the region interacting with IAP do not inhibit platelet aggregation induced by several agonists[158]. We have now demonstrated a role for platelet-secreted TSP in microaggregate formation at laminar shear flow rates between G = 300 -2000 s^{-1}, for platelets activated with threshold concentrations of thrombin (Chapter 5). Immobilized TSP has previously been shown to support adhesion in a Ca^{2+}-dependent manner. Agbanyo et al[147] have shown that while adhesion of platelets from whole blood to Ca^{2+}-depleted immobilized TSP shows anti-adhesive properties, adhesion to Ca^{2+}-replete TSP can support platelet adhesion, with maximum efficiency occurring at G = 1500 s^{-1}, and dropping sharply with increasing G.

5.3. Fibronectin[14]

Fibronectin (FN) is a large asymmetric molecule of MW 500,000 Da with globular domains, and composed of two similar subunits which are disulfide linked. It can be synthesized by a variety of cells in vitro including fibroblasts, endothelial cells, smooth muscle cells and epithelial cells. It is also incorporated into platelet alpha granules, and released upon platelet activation. It plays an important role in cell adhesion, morphology and cytoskeletal organization, and is involved in interactions of cells with the extracellular matrix. FN has binding sites for collagen, heparin, actin, F. XIII and fibrin. Thus, FN actually becomes covalently linked to fibrin by F. XIII*, providing sites for cells involved in tissue repair, such as fibroblasts, and for adhesion to the clot. There is also an RGDS site, which may be recognized by platelet GPIIb-IIIa or VN receptor $\alpha_V\beta_3$. Platelet adhesion to

immobilized FN is supported under static conditions, and under flow conditions. Under flow conditions, purified FN mediates platelet adhesion optimally G at 300 s^{-1}, and promotes only ≤5% surface coverage at G =1000 s^{-1} [159]. However, one study suggests subendothelial FN may be important even at G = 1300 s^{-1} [62]. The adhesion observed is dependent upon vWF and GPIb[74].

5.4. Laminin [14]

Laminin is the major non-collagenous glycoprotein component of basement membranes, where it binds preferentially to Type IV collagen[160]. It is a very large molecule (MW approximately 800 kDa), composed of at least three protein chains associated by disulfide linkages. Rotary shadowing electron microscopy depicts it as a twisted cruciform structure with three short arms and a long arm, with globular domains at extremities of each arm. This form is similar to that observed with atomic force microscopy[161], where the molecule was assigned a length of approximately 50nm and 76 nm, for the short and long arms, respectively. Additionally, they showed that there was considerable flexibility of the arms, suggesting movement. Laminin has been shown to play a role in cell attachment, cell growth, tissue development and differentiation. Adhesion to purified laminin requires Mg^{2+} and Ca^{2+}, and is shear-rate dependent, with maximum coverage occurring at G = 800 s^{-1} [162].

6. PLATELET AGONISTS [163]

6.1. Adenosine diphosphate (ADP)

Adenosine diphosphate (ADP) is a physiological activator of platelet GPIIb-IIIa, and

causes one or all of: platelet shape change, reversible aggregation[164], and alpha and dense granule release, in a concentration-dependent manner[165]. Calcium is required for ADP-mediated aggregation and secretion. Using antagonists of ADP such as ticlopidine, clopidogrel[166] or apyrase[167], ADP was shown to be physiologically relevant in the recruitment of platelets in thrombosis. It is also required for shear-induced platelet aggregation (SIPA) [168].

There are two pools of nucleotides in platelets (comprising ADP and ATP): within dense granules or within a cytoplasmic pool. Only nucleotides in the cytoplasmic pool are readily labelled, suggesting that there is no exchange between pools[169]. The ADP concentration in dense granules is approximately 653 mM, while for ATP it is 436 mM [170], and these may be released upon platelet activation. Upon adhesion of platelets to exposed matrix at sites of vascular injury, platelets become activated and may secrete alpha and dense granule contents, thereby activating nearby platelets in solution and amplifying the aggregation response. Shear rates of $G \geq 5000\ s^{-1}$, may induce leakage of platelet stored ADP[54].

Platelet activation responses (shape change, secretion, receptor activation) are energy dependent and consume ATP through enzymatic hydrolysis[170], to yield primarily ADP and AMP for conversion to cAMP. Presumably some ADP generated by degradation of ATP may also contribute to activation of platelets, since the $t_{1/2}$ for ATP in plasma is approximately 1.5 minutes, while for ADP in plasma it is approximately 4 minutes. ADP is converted to AMP, then to adenosine, which inhibits platelet activation and can shut down the platelet response. Erythrocytes may also release ADP for platelet activation, from

damaged or fragmented cells, presumably by compression experienced when flowing past stenotic regions. A physiologic role for RBC's may be postulated based on the increase in bleeding time (reflecting status of primary hemostasis) in patients with low hematocrits [172].

6.1.1. Receptors and signalling

Although ADP binding to platelet surfaces has been acknowledged for some time, identification of the receptors involved in ADP-mediated platelet responses has happened only recently. Some of the confusion stemmed from the effect of ADP on up regulation of both adenylyl cyclase, and of platelet activation and aggregation in flow, and has been resolved by the discovery of at least two distinct G-protein coupled receptors of the family of P_2 purinergic receptors (classified by their preference for a variety of nucleotide analogue agonists, versus ones recognizing adenosine (P_1 receptors)). These have been determined using antibodies against the cloned $P2Y_1$ receptor, as well as competitive inhibitors such as 2-methylthio-AMP (2MeSAMP). The first receptor, $P2Y_1$, coupled to G_q and phospholipase C, activates mobilization of $[Ca^{2+}]_i$ and mediates shape change[173]. The second receptor ($P2_{AC}$)is coupled to G_j and is inhibited by ticlopidines and ATP. It mediates the decrease in c-AMP levels, and inhibits the binding of [^{3}H] -2-MeS-ADP. Both receptors are required for mediating platelet aggregation. A third potential ADP receptor, P2X1, has been identified and its function remains to be characterized[174].

ADP also induces cytoskeletal changes in platelets required for shape change and activation of GPIIb-IIIa receptors[175,176], with PI3Kinase involved in the signaling pathway [174]. Several studies have suggested that ADP removal from its receptors can reverse GPIIb-

IIIa activation, suggesting a requirement for sustained signaling. Activation of platelets with ADP can result in the downstream production of DAG and IP3[177], with subsequent liberation of arachidonic acid (a platelet agonist), and production of eicosanoids (thromboxanes) which are extremely strong agonists. ADP stimulation may also cause an increase in cytosolic pH mediated by a Na+/H+ exchanger, a transient rise in free Ca^{2+} concentration, and phosphorylation of myosin light chain[178]. Activation of protein kinase C does not appear to play an important role in the primary phase of ADP-induced aggregation of human platelets [179].

6.2. Thrombin[180,181]

Thrombin is one of the strongest and most important agonists known. It can regulate both fibrinolytic (activation of protein C), and pro-coagulant responses (activation of hemostatic clot cascade proteins F. V, F. VIII, F. XIII) via its serine protease activity. The precursor for thrombin, prothrombin, is converted to the active form through the sequential activation of clotting cascade proteins, culminating in the assembly of the prothrombinase complex to yield thrombin, on activated platelets[182], endothelial cells[183], and monocytes[184]. Thrombin can amplify its own generation via a positive feedback circuit[185]. Locally formed thrombin then cleaves fibrinogen, with release of fibrinopeptides A and B, thereby initiating fibrin polymerization, required for formation of a stable platelet plug for hemostasis. Additionally, thrombin can activate platelets, neutrophils, endothelial cells and smooth muscle cells, and promote both mitogenic and migratory responses. Due to its many functions, thrombin has the potential to greatly influence major events in the thrombotic

process. As such, much effort has gone into the development of antagonists against it.

6.2.1. Thrombin structural domains

Thrombin is a 37 kDa. Protein. It is an ellipsoid of approximately 45x45x50 Å. There are two positively-charged anion-binding domains, referred to as Exosites I and II, which largely determine initial recognition of substrates by thrombin. A proteolytic site found in a large groove, is defined by the catalytic triad consisting of His 57, Ser195 and Asp102. Fibrinogen, fibrin monomer[186], and a seven-transmembrane thrombin receptor[187] and the thrombin antagonist, hirudin, have been shown to bind to exosite I. Exosite II is the site of binding for heparin[188], chondroitin sulfate[189] and prothrombin fragment SPII[190]. The exosite which interacts with GPIb has not been definitively determined, as evidence for binding to either exosite exists[191,192]. Additionally, small substrates may bind directly to the active site. Peptides corresponding to the carboxyl domain of hirudin have been used to show that for larger proteins, the first interaction between substrate and protein is via the exosites, required to modulate affinities for interaction with the catalytic site[193]. Labeled peptides have been used to show that allosteric changes may be induced in the active site of the molecule following engagement of exosites[194]. Similarly binding of hirudin peptides to Exosite I can displace prothrombin SPII fragments from Exosite II, and vice versa, suggesting reciprocally regulated conformation of exosites[195].

6.2.2. Thrombin platelet receptors

Thrombin can bind to at least two types of receptors on platelet surfaces (Fig. 1-5). There is ample evidence showing a direct interaction between glycoprotein receptor GPIb and thrombin (see section in glycoprotein receptor GPIb). GPIb is generally considered to

be the high affinity receptor for thrombin, with a K_d of 0.3 - 0.5 nM, and with approximately 50 -200 sites per platelet. Two protease-activated receptors (PAR) have also been detected on human platelets; PAR-1[196] and PAR-4[197]. PAR-1 is expected to be the medium affinity receptor for thrombin, with a Kd of approximately 2 - 40 nM, and 200 - 2000 sites per platelet[198]. The Kd for thrombin binding to PAR-4 is an order of magnitude greater[199]. The existence of an additional receptor for mediating high affinity binding has been proposed[200].

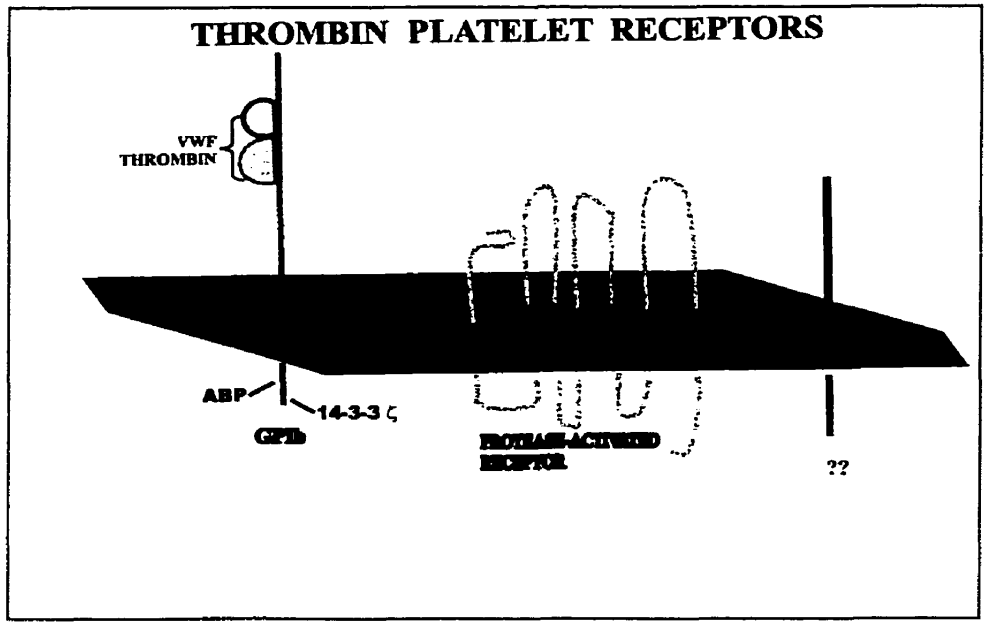

Fig. 1-5.Thrombin platelet receptors. Thrombin can bind to at least two types of binding sites on human platelets: 1) GPIb, where the binding site is immediately adjacent to but distinct from the vWF binding site, and 2) members of the protease-activated receptor family, PAR-1 and PAR-4. A third distinct receptor has been proposed by Hayes et al to function as the high affinity receptor and is thus far uncharacterized.

A great deal of controversy is associated with assignment of degree of affinity of thrombin for these receptors. Monoclonal antibodies against GPIb sites, or proteolysis by Serratia marcescens protease (which preferentially cleaves GPIb over PAR-1), selectively reduce intracellular Ca^{2+} mobilization induced by low thrombin concentrations[198]. However, the B_{max} expected to reflect numbers of high affinity binding sites, is very low compared to total GP Ib receptors reported on the platelet surfaces (~25,000). The existence of a subclass of GPIb receptors which mediates thrombin interactions, possibly in a dimerized state, has been proposed to account for this discrepancy[201]. Alternatively, moderate affinity binding to glycocalicin (a soluble extracytoplasmic portion of GPIb comprising the thrombin binding site) has been described. This affinity was similar to that determined for thrombin binding to platelets at 4^0 C rather than at 37^0 C, where binding is fully reversible, suggesting that previous determinations of K_d's had not been done under true equilibrium binding conditions, and obviating GPIb's high affinity status[202].

A very recent report has suggested that full platelet activation responses can be accounted for by PAR-1 and PAR-4, as full blockage of responses could be achieved using antibodies against these two receptors alone[199]. However, some cross-inhibition of thrombin high and moderate affinity responses occurs by antagonists directed against either receptor. Peptides against GPIb have been shown to inhibit low thrombin-concentration-mediated activation as well as responses mediated by SFLLRN (thrombin activating peptide, derived from PAR-1 sequences immediately post cleavage site)[203]. Similarly, antibodies against PAR-1 can inhibit low thrombin activation responses[204]. Thus, there is an additional concern over antagonist specificity. Nevertheless, some strong evidence in favour of the glycoprotein

GPIb receptor as the high affinity thrombin platelet receptor, stems from studies comparing Bernard Soulier syndrome patients, having giant platelets and lacking the GPIb receptor, with May-Hegglin patients, having giant platelets but normal GPIb. In these experiments, aggregation in response to low concentrations of thrombin was greatly decreased with BSS patient platelets, despite normal or increased surface expression of PAR-1 receptors on platelet surfaces[205]. Additionally, surviving knock-out mice lacking the PAR-1 receptor appear to have relatively normal hemostatic function[206], corroborating the role of other thrombin surface receptors. At any rate, we have shown that qualitative differences in platelet function occur at discrete thrombin-concentrations, also consistent with the existence of more than one receptor mediating platelet responses induced by thrombin (Chapter 4, 5).

6.2.3. Signalling via thrombin receptors

Platelet thrombin receptors are associated with signaling molecules, as well as with the cytoskeleton. The cytoplasmic tail of glycoprotein GPIb is associated with the cytoskeleton via actin binding protein (ABP), and with a phospholipase A2, 14-3-3 zeta isoform as discussed in section 7.1 on glycoprotein GPIb. Thus activation of platelets through GPIb may proceed via liberation of arachidonic acid, and activation of p42/44 ^{mapk} and pp60^{src} [201]. Downregulation of GPIb receptors upon activation with increasing thrombin concentrations may occur at low Ca^{2+} levels[207,208]. Signaling transduction mediated by the PAR-1 receptor is associated with activation of phospholipase C, generation of inositol phosphates and activation of protein kinase C[209]. Differences in activated protein kinase C signalling at low (0.05 U/ml) versus high thrombin concentrations (0.5 U/ml), have been invoked to explain

differences in maintenance of the activated state of unoccupied GPIIb-IIIa receptors [210].

6.3. Epinephrine

Epinephrine may be released into the circulation from the human adrenal medulla during stress[211]. Increased catecholamine levels in early morning have been correlated with the frequency of onset of myocardial infarction, and an increase in platelet aggregability [212,213]. Epinephrine can inhibit adenylyl cyclase[214] by binding to α_2-adrenergic receptors, which are linked to adenylate cyclase, and coupled to a G-protein[215]. There are approximately 270 receptors per platelet, and binding occurs with a K_d of 2.5 nM[216].

The response to epinephrine by platelets is highly variable. Epinephrine has been shown to synergize with other agonists such as ADP[217], or high shear[218] to activate platelets. In fact, in previous studies where epinephrine was thought to have induced aggregation, ADP present in platelet preparations was likely a co-agonist, since for platelets washed in the presence of prostacyclin and resuspended in buffer containing apyrase, no shape change, serotonin secretion or macroaggregates were detectable in response to epinephrine[219]. However, relatively low epinephrine concentrations (20 nM), have been shown to induce microaggregate formation, redistribution of filamentous actin, and GPIIb-IIIa activation[220]. A functional chloride transport system is also required for epinephrine-mediated aggregation [221]. At higher epinephrine concentrations (4 μM), low levels of protein tyrosine phosphorylation, and Syk activation occurred. Moreover, epinephrine down-shifted the thrombin concentrations required to promote more extensive signaling responses, as well as platelet aggregation[222]. Treatment with the tyrosine kinase inhibitor, genistein, inhibited the

synergistic effect of epinephrine. In hypercholesterolemic patients, epinephrine may induce significantly greater platelet aggregation than in control subjects[223].

6.4. Thromboxanes

The thromboxanes are part of the family of eicosanoids, which are newly synthesized in response to specific agonists or physical perturbation, from arachidonic acid liberated from platelet membranes by phospholipases. Thromboxane A2 (TxA2) is extremely labile, with a $t_{½}$ of 30 minutes in protein-free aqueous media[224]. It is in turn enzymatically converted to Thromboxane B2, which is stable, but without known biological activity. TxA2 inhibits the stimulation of platelet adenylate cyclase, thus lowering cAMP. Its production can be inhibited by cyclo-oxygenase antagonists such as indomethacin and by nonsteroidal anti-inflammatory drugs like aspirin[225].

Thromboxane may induce its biological effects by promoting dense granule secretion of ADP[226], since a full aggregation response cannot be obtained upon enzymatic removal of ADP. Nevertheless, endoperoxides can potentiate aggregation induced by an arachidonic acid derivative, in patients with storage pool disease, where storage granules lack ADP[227]. An increase in thromboxane metabolites has been observed in patients with unstable ischemic coronary syndromes[228], suggesting a role for thromboxane in thrombosis.

7. GLYCOPROTEIN RECEPTORS

7.1. Glycoprotein (GP) Ib[229,230]

The glycoprotein receptor, GPIb, is composed of two disulfide-linked chains, GPIbα and GPIbß, with approximately 25,000 receptors detectable on platelet surfaces. It is tightly but not covalently associated with GPIX and less strongly with GPV. Members of the complex are present in a ratio of 2:1:2 (GPIb(α,ß): GPV:GPIX). Molecular weights are 145, 22, 82 and 17 kDa respectively. All are transmembrane proteins with large extracytoplasmic domains and short cytoplasmic tails. Approximately 70 % of the total GPIb is linked to the platelet membrane cytoskeleton via actin binding protein (also known as filamin)[231], with which GPIb associates through its cytoplasmic tail residues Thr 536 - Phe 568 [232]. This association apparently does not require any externally added platelet activators. A phospholipase A2 (14-3-3 zeta isoform) is associated with the last five residues of the carboxyl domain of GPIbα[233,234] and interacts with GPIbß as well[235]. All members of the complex have in common a leucine rich region (LRR) which may be involved in protein-protein interactions. X-ray crystallography studies with ribonuclease inhibitor[236] suggest that by homology, these regions may form an arc in GPIbα (6 LRR repeats), and a horseshoe in GPV (15 LRR repeats), and may be important in maintaining the structural integrity of the receptor. These LRR are found in the proximal region of the amino terminal 45kDa domain, anterior to two disulfide-linkage determined loops representing binding sites for vWF and thrombin.

Rotary shadowed electron microscopy studies by Fox et al[237], have depicted GPIb as a flexible rod with a globular domain at either end. The total length is approximately 60 nm,

with approximately 50 nm representing the extracytoplasmic domain. There is extensive glycosylation, mostly found in the 84 kDa macroglycopeptide region, where it accounts for about 59% of the weight, on a molar basis[238]. The dense carbohydrate coverage is thought to provide a barrier to macromolecules, and to maintain ligand domains extended well beyond the platelet surface. Proteases which cleave GPIb close to its membrane insertion site, release the large extracellular portion, referred to as glycocalicin[239,240]. Glycocalicin can be found circulating in plasma at concentrations between 1-3 μg/ml[241], suggesting that there is ongoing catabolism of GPIb in vivo.

Under normal circumstances, correct intracellular assembly of GPIbα and optimal surface expression of the complex is dependent upon GP IX and GPIbß[242,243], although GPIbα can be singly expressed on model cells when linked to ICAM-1[244]. This surface-expressed GPIbα can support ristocetin- or botrocetin-mediated binding of vWF in the absence of other complex members. In platelets, GPIb can be found associated with the plasma membrane, OCS and α-granule membranes[245]. Several groups have detected GPIb on endothelial cells in vivo and in vitro[246,247], with all four members of the complex (GPIb α,ß, GPV, GPIX) being expressed in the same ratios as on platelets[248]. However, mRNA or endothelial surface expression of GPIb were not detected by at least one group[249], suggesting that differences in experimental conditions may influence detectability of this protein.

The members of the GPIb-IX-V complex are susceptible to, and may be individually regulated by proteolysis. GPV may be cleaved by thrombin during activation with release of a 69 kDa fragment[250], while GPIbα may release glycocalicin upon cleavage by neutrophil cathepsin G [251]. Both agonists induce reversible redistribution of the remainder of the

complex from the platelet surface to membranes of the OCS. Such cleavage events may occur during close contact between platelets and neutrophils during thrombosis or inflammation events.

There are three bleeding disorders associated with deficient or defective GPIb: Bernard Soulier syndrome, platelet-type von Willebrand disease and velocardiofacial syndrome[252]. Bernard Soulier syndrome (BSS) is characterized by thrombocytopenia, giant platelets, and functionally defective, or almost absent GPIb receptors[253], suggesting a link between megakaryocytopoiesis and GPIb expression. Several BSS mutations occur either in the LRR of GP IX or in GPIbα[229,254], suggesting that structural modifications may result in inappropriate processing of the molecule. In platelet type vWD, single amino acid substitutions within a narrow region of GPIbα, yield a protein with affinity for vWF sufficiently increased to allow interactions to occur in the absence of additional agonists[255], thereby promoting intravascular clumping.

7.1.1.GPIb function

The principal adhesive ligand of the glycoprotein Ib receptor is von Willebrand factor. Thus, platelet adhesion is mediated by two sources of vWF: one is present within the extracellular matrix, exposed upon vascular injury, and a second is plasma vWF adsorbed onto matrix collagens. GPIb and vWF have been implicated in thrombin generation[185] and in promoting platelet incorporation into polymerizing fibrin[99,101]. The GPIb receptor is also required for mediating shear-associated processes such as the aggregation of weakly

activated platelets[256] (Chapters 2-5), and the rolling of platelets on immobilized purified vWF [96]. In the latter case, an intact GPIb-IX-V complex is necessary[257].

As mentioned previously, there is a binding site for thrombin on GPIb, to which thrombin binds with a K_d of 0.3 - 0.5 nM, and a B_{max} of 50-200 sites [198,258]. However, the status of GPIb as the thrombin high-affinity receptor has lately been questioned based on irreversibility of the GPIb-thrombin interaction and affinities for glycocalicin[202]. Other experiments by Hayes et al[200], suggest that platelet surface localizations of GPIb and of anti-hirudin peptide antibodies are different, where a hirudin carboxyl terminus peptide is expected to represent the high-affinity site. The full expression of the GPV receptor, although not itself expected to constitute the binding site for thrombin, is nevertheless required for highest affinity binding[259], perhaps accounting for differences in thrombin affinity for glycocalicin versus for the intact complex on platelet surfaces.

Studies using monoclonal antibodies and peptides suggest that epitopes associated with thrombin binding encompass a large region circumscribed by residues 216 and 285. This overlaps regions important for vWF binding, which occur between residues 233 and 287 [260]. Antibodies exist which inhibit GPIb interactions with both (e.g. TM-60 [261]) or only one of [262,263] thrombin and vWF. Snake venom-derived proteins which recognize GPIbα, may also regulate binding of ligands and aggregation mediated by this receptor. Thus, alboaggregins can bind to platelets and induce platelet agglutination, as well as competitively inhibit vWF binding to GPIb[264]. Echicetin, similarly to some antibodies, affects several GPIb functions; inhibiting thrombin binding to GPIb, and platelet aggregation mediated by alboaggregins, or botrocetin and soluble vWF[265,266]. Mutations in the LRR region of GPIbα can also disrupt

vWF-GPIb interactions[267], as indicated by studies of human-dog chimeras. This may occur by affecting the structural integrity of the receptor, or by directly disturbing a section of the binding epitope for vWF.

7.2. Glycoprotein (GP) IIB-IIIa [253,268]

Glycoprotein IIB-IIIa ($\alpha_{IIb}\beta_3$) is a member of the integrin family, a group of receptors important in mediating adhesive interactions. GPIIb-IIIa is specifically required for platelet aggregation, firm adhesion, spreading and clot retraction, and participates in platelet-leukocyte interactions[269]. As such, it has been assessed as a therapeutic target against thrombosis. Defective or absent GPIIb-IIIa leads to a bleeding disorder, Glanzmann's thrombasthenia, characterized by variably severe hemorrhaging, absent clot retraction and absent platelet aggregation in response to several agonists.

GPIIb-IIIa is the most abundant integrin on platelet surfaces, with approximately 50,000 uniformly distributed molecules detectable on the surface of resting platelets, with $\leq$ 100,000 on maximally activated platelets[270]. GPIIb-IIIa is a heterodimer of non-covalently associated α_{IIb} and β_3 chains. It shares the β_3 subunit with the vitronectin receptor, $\alpha_V\beta_3$. The alpha chain (αIIb or GPIIb), is a disulfide-linked dimer consisting of a heavy chain ($GPIIb_\alpha$-125 kDa) and a light chain ($GPIIb_\beta$-25 kDa). The transmembrane domain and a small cytoplasmic domain (26 residues) are found within the light chain. In the heavy chain, there are four extracellular cation binding sites, which are required for most ligand interactions. The beta chain (β_3 or GPIIIa; 105 kDa) also has a transmembrane domain. There are 56 cysteine residues in the intact complex, yielding a tight and globular molecule. Electron microscopy studies have indicated that the structure of GPIIb-IIIa includes a globular head domain of

approximately 10 nm, and two 18 nm flexible tails[271,272]. Association between α and ß subunits is cation dependent, and required for receptor ligand recognition, mediated by the N-terminal region of both subunits[273]. Peptides derived from the cytoplasmic tails of α_{IIb} and $ß_3$ may also interact, shown using fluorescence quenching and terbium luminescence techniques[274].

7.2.1. Activation-dependent changes and ligand binding

GPIIb-IIIa may be considered to exist in at least three conformational states: unactivated, activated but not ligand occupied and, activated, ligand occupied . Thus, upon activation, GPIIb-IIIa undergoes a conformational change in the extracellular domain (detectable using activation-dependent antibody PAC-1[275], and resonance energy transfer[276]. It thereby greatly increases its affinity towards, and becomes competent to bind, soluble Fg as well as other RGD-containing adhesive ligands vWF, vitronectin (VN), and fibronectin (FN). Subsequent to ligand binding, new epitopes are exposed on the receptor (ligand-induced binding sites, LIBS), and may be detected by monoclonal antibodies. Upon activation with thrombin, ligand binding may even occur directly within granules, as detected using monoclonal antibody AP-6 [277].

Unactivated GPIIb-IIIa can only recognize small peptides, or molecules containing an RGD, KDG or RYD sequence positioned at the tip of a recognition loop, or immobilized on a bead, and protruding 14 - 32 Å from the protein core[278,279]. Thus, the binding pocket of unactivated GPIIb-IIIa is postulated to resemble a narrow cavity buried 10-20 Å within the protein, and to interact with larger molecules solely upon agonist-induced changes in its quaternary structure. Specific recognition sites for the dodecapeptide sequence of Fg on

GPIIb-IIIa have been cross-linked to residues 294-314 on GPIIb[280], whereas the ubiquitous RGD sequence has been cross-linked to residues 109-171 on GPIIIa[281] (Fig. 1-6). A peptide derived from residues 211-222 on GPIIIa has also been shown to inhibit Fg binding[282]. Binding of GRGDSP peptide and γ-chain dodecapeptide to intact GPIIb-IIIa complex, however, are mutually exclusive, suggesting that these two regions are spatially or conformationally related[283]. Alternatively, it has been suggested that there may be two GPIIb-IIIa -ligand binding pockets, which are allosterically associated[284].

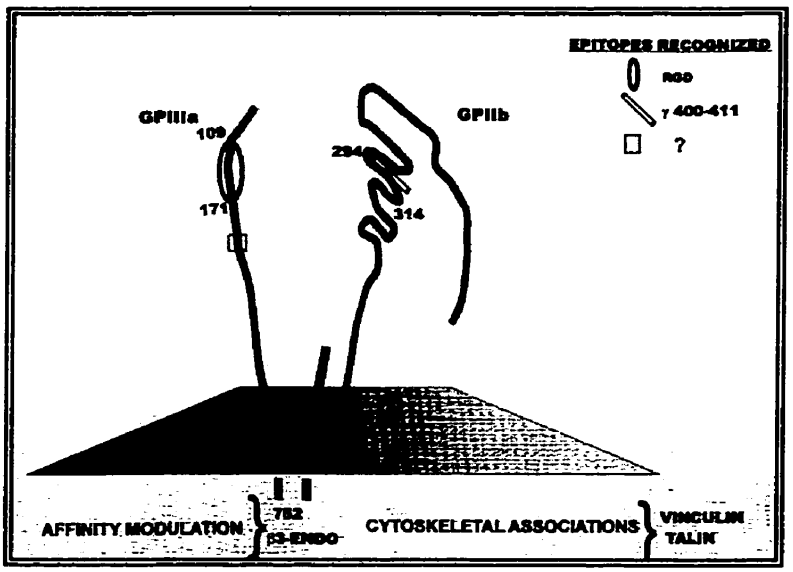

Fig. 1-6. Activated GPIIb-IIIa receptor. Following activation, GPIIb-IIIa can recognize RGD sequences in adhesive ligands as well as the gamma terminus of Fg. A fraction of GPIIb-IIIa receptors also become associated with the membrane skeleton. The cytoplasmic tail of GPIIb-IIIa is involved in the regulation of its affinity state, with the binding of ß-3 endonexin (ß3-ENDO) positively and mutations at residue 752, negatively regulating affinity state.

7.2.2. Regulation of affinity; signaling complexes

GPIIb-IIIa receptor ligand interactions are regulated by affinity modulation, transduced by signaling molecules following agonist activation, or by accessibility to conformationally appropriate ligand recognition sequences, as discussed above. The process leading to receptor activation following agonist binding to platelets, is referred to as "inside-out" signaling. Additional changes following ligand binding result in "outside-in" signaling. The specific signal-transducing molecules are incompletely known. However, activation by different agonists appears to converge in a common pathway, possibly the phosphorylation of protein kinase C[210,285], to lead to GPIIb-IIIa activation. Investigation of specific receptor domains involved in each signaling process indicates that mutations in the cytoplasmic tail can alter binding affinity. Thus, the point mutation S752P in GPIIIa has been shown to block inside out signaling, and to prevent receptor activation[286]. In contrast, β_3-endonexin binding to GPIIIa can positively regulate GPIIb-IIIa affinity state, as shown using transfected CHO cells. The increased affinity state could be inhibited by co-expression of the β_3 tail or H-ras, thus indicating that second messengers were likely involved in affinity upregulation[287]. Structural mutations in α or β cytoplasmic domains, affecting a salt bridge in a hinge region comprised of both chains, can alter inside out signaling and yield GPIIb-IIIa in a permanently activated state[288]. A functional sodium/calcium exchanger also appears to function in inside-out signaling [289], as inhibitors of this exchanger reduced agonist-induced platelet aggregation. Phosphorylation of pleckstrin or myosin light chain were not affected. Antibody binding to LIBS epitopes or to resting GPIIb-IIIa may also cause increases in cytosolic Ca^{2+} levels[290] or kinase activity[291]. Signaling may be attenuated by phosphatases.

A fraction of the GPIIb-IIIa in resting platelets is associated with cytoskeletal proteins talin, and vinculin[292]. However, approximately 50 % of receptors in megakaryocytes move freely within the plane of the membrane, shown by fluorescence-recovery after photobleaching[293]. Upon activation, another 25 % become immobilized. This is likely due to a transfer of GPIIb-IIIa from the membrane skeleton to associations with cytoplasmic actin filaments[294].

GPIIb-IIIa receptors may be activated and remain monodisperse[295]. Clustering of GPIIb-IIIa receptors, occurs only after ligand binding[294], thereby increasing the valency, and therefore avidity of the reaction. Events subsequent to ligand binding and aggregation include outside-in signaling[296,297], activation of calpain[298] and formation of focal-adhesion-like complexes[292], similar to contacts formed between cells and extracellular matrix. Different signaling molecules may be implicated following activation alone, as compared to after aggregation. The signaling molecule ppSYK becomes phosphorylated following ligand binding, whereas pp125 FAK becomes phosphorylated only after ligand binding and platelet aggregation[299]. Different signaling pathways may affect the stability of activated GPIIb-IIIa conformations, depending on agonist and concentration. Thus, GPIIb-IIIa receptors on platelets activated with ADP or low thrombin concentrations (0.05 U/ml), will maintain their activated conformation for only a few minutes in the absence of bound ligand before reverting to the resting conformation, and are not resistant to closure of sites by PGI_2. This is in contrast to activation at higher doses of thrombin (0.5 U/ml), where sites remain open for at least 20 minutes[210].

The targeting sequence NPXY on the cytoplasmic region of GP IIIa has been postulated

to participate in the regulation of integrin affinity states[300], and in the internalization of Fg and vWF through clathrin coated-pits[118]. The co-immunoprecipitation of GPIIb-IIIa with AP-2 adaptor proteins, involved in the formation of clathrin-coated vesicles, suggests a mechanism for vesicle-mediated trafficking of this receptor[301]. Loss of receptor due to internalization may also regulate ligand interactions.

8. REFERENCES:

1. Savage B, Almus-Jacobs F, Ruggeri ZM. Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. Cell 1998; 94: 657-66.

2. Suzuki H, Kinlough-Rathbone RL, Packham MA, Tanoue K, Yamazaki H, Mustard F. Immunocytochemical localization of fibrinogen during thrombin-induced aggregation of washed human platelets. Blood 1988; 71: 1310-20.

3. Ofosu FA, Longbin L, Freedman J. Control mechanisms in thrombin generation. Sem Thromb Hemost 1996; 22: 303-8.

4. Falk, E, Fernandez-Ortiz A. Role of thrombosis in atherosclerosis and its complications. Am J Cardiol 1995; 75: 5B-11B.

5. Ghigliotti G, Waissbluth AR, Speidel C, Abedschein DR, Eisenberg PR. Prolonged activation of prothrombin on the vascular wall after arterial injury. Arterioscler Thromb Vasc Biol. 1998; 18: 250-7.

6. Sevitt S. The structure and growth of valve-pocket thrombi in femoral veins. J Clin Pathol 1974; 27: 517-28.

7. Yamamoto H, Vreys I, Stassen JM, Yoshimoto R, Vermylen J, Hoylaerts MF. Antagonism of vWF inhibits both injury induced arterial and venous thrombosis in the hamster. Thromb Haemost 1998; 79: 202-10.

8. Bini A, Fenoglio JJ Jr, Mesa-Tejada R, Kudryk B, Kaplan KL. Identification and distribution of fibrinogen, fibrin and fibrin(ogen) degradation products in atherosclerosis. Arteriosclerosis 1989; 9: 109-21.

9. Wight TN, Raugi GJ, Mumby SM, Bornstein P. Light microscopic immunolocation of

thrombospondin in human tissues. J Histochem Cytochem 1985; 33: 295-302.

10. Schwartz SM, Majesky M. Structure and function of the vessel wall. In: Hemostasis and Thrombosis: Basic principles and practice, third ed 1994; Colman RW, Hirsh J, Marder VJ, Salzman EW (eds). J.B. Lippincott Co, Philadelphia.

11. Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae KR, Hug BA, Schmidt AM, Stern DM. Endothelial cells in physiology and in pathophysiology of vascular disorders. Blood 1998; 91: 3527-61.

12. Augustin HG, Kozian DH, Johnson RC. Differentiation of endothelial cells: analysis of the constitutive and activated endothelial cell phenotypes. Bioessays 1994; 16: 901-6.

13. Hatton MWC, Berry LR, Regoeczi E. Inhibition of thrombin by antithrombin III in the presence of certain glycosaminoglycans found in the mammalian aorta. Thromb Res 1978; 13: 655-70.

14. Kefalides NA. The biochemistry and molecular biology of extracellular matrix. In: Hemostasis and Thrombosis: basic principles and clinical practice, third ed 1994; Colman RW, Hirsh J, Marder VJ, Salzman EW (eds). J.B. Lippincott Co, Philadelphia

15. Ramachandran GN. Stereochemistry of collagen. Intnal J Peptide Peptide Res 1988; 31: 1-16.

16. Tanzer ML. Collagens and elastins: structure and interactions. Curr Opinion Cell Biol 1989; 1: 968-73.

17. Van der Rest M, Garrone R. Collagen family of proteins. FASEB J 1991; 5: 2814-23.

18. Rauterberg JE, Jaeger J. Collagen and collagen synthesis in the atherosclerotic vessel

wall. In: Cell Interactions in Atherosclerosis, 1992; Robenek H, NJ Severs (eds). CRC Press, Boca Raton 1992, pp 102-35.

19. Bork P. The molecular architecture of vertebrate collagens. FEBS Lett 1992; 307:49--54.

20. Rauterberg J, Jander R, Troyer D. Type VI collagen-a structural glycoprotein with a collagenous domain. Front Matrix Biol 1986; 11:90-109.

21. Engvall E, Hessle H, Klier G. Molecular assembly, secretion, and matrix deposition of Type VI collagen. J Cell Biol 1986; 162: 703-10.

22. Rand Jh, Wu XX, Potter BJ, Uson RR, Gordon RE. Co-localization of von Willebrand factor and type VI collagen in human vascular subendothelium. Amer J Pathol 1993; 142: 843-50.

23. Keene DR, Engvall E, Glanville RW. Ultratructure of Type VI collagen in human skin and cartilage suggests an anchoring function for this filamentous network. J Cell Biol 1988; 107: 1995-2006.

24. Nieuwenhuis HK, Akkerman JWN, Houdijk WPM, Sixma JJ. Human blood platelets showing no response to collagen fail to express surface glycoprotein GPIa. Nature 1985; 318: 470.

25. Saelman EUM, Nieuwenhuis HK, Hese KM, de Groot PG, Heijnen HFG, Sage EH, Williams S, McKeown L, Gralnick HR, Sixma JJ. Platelet adhesion to collagen Types I through VIII under conditions of stasis and flow is mediated by GPIa/IIa ($\alpha 2\beta 1$-integrin). Blood 1994; 83:1244-50.

26. Heemskirk JWM, Siljander P, Vuist WMJ, Breikers G, Reutelingsperger CPM, Barnes MJ, Knight CG, Lassila R, Farndale RW. Function of glycoprotein VI and integrin $\alpha_2\beta_1$ in

the procoagulant response of single, collagen-adherent platelets. Thromb Haemost 1999; 81:782-92.

27. Diaz-Ricart M, Tandon NN, Carretero M, Ordinas A, Bastida E, Jamieson GA. Platelets lacking functional CD 36 (glycoprotein IV) show reduced adhesion to collagen in flowing whole blood. Blood 1993; 82: 491-6.

28. Moroi M, Jung SM, Shinmyozu K, Tomiyama Y, Ordinas A, Diaz-Ricart M. Platelet adhesion to a collagen-coated surface under flow conditions: The involvement of glycoprotein VI in the platelet adhesion. Blood 1996; 88: 2081- 92.

29. Houdijk WPM, Sakariassen KS, Nievelstein PFEM, Sixma JJ. Role of factor VIII-von Willebrand factor and fibronectin in monomeric and fibrillar human collagen Types I and III. J Clin Invest 1985; 75: 531-40.

30. Morton LF, Hargreaves PG, Farndale RW, Young RD, Barnes MJ. Integrin α2ß1-independent activation of platelets by simple collagen-like peptides: collagen tertiary (triple-helical) and quaryternary (polymeric) structures are sufficient alone for α2ß1-independent platelet reactivity. Biochem J 1995; 306: 337-44.

31. Verkleij MW, Morton LF, Knight CG, de Groot PG, Barnes MJ, Sixma JJ. Simple collagen-like peptides support adhesion under static but not under flow conditions: interaction via α2ß1 and von Willebrand factor with specific sequences in native collagen is a requirement to resist shear forces. Blood 1998; 91: 3808-16.

32. Barstad RM, Roald HE, Cui Y, Turitto VT, Sakariassen KS. A perfusion chamber developed to investigate thrombus formation and shear profiles in flowing native human blood at the apex of well-defined stenoses. Arterioscler Thromb 1994; 1984-91.

33. Mazzucato M, Spessotto P, Masotti A, De Appollonia L, Cozzi MR, Yoshioka A, Perris R, Colombatti A, De Marco M. Identification of domains responsible for von Willebrand factor Type VI collagen interaction mediating platelet adhesion under high flow. J Biol Chem 1999; 274: 3033-41.

34. Asselin J, Gibbins JM, Achison M, Lee YH, Morton LF, Farndale RW, Barnes MJ, Watson SP. A collagen-like peptide stimulates tyrosine-phosphorylation of syk and phospholipase $C\gamma_2$ in platelets independent of the integrin $\alpha_2\beta_1$. Blood 1997; 89: 1235-42.

35. Oka S. Cardiovascular Hemorheology.Cambridge, 1981. University Press, Cambridge.

36. Goldsmith HL, Turitto VT. Rheological aspects of thrombosis and haemostasis: basic principles and applications; ICTH - Report - Subcommittee on rheology of the international committee on thrombosis and haemostasis. Thrombos Haemost 1986; 55: 415-35.

37. McDonald DA. Blood flow in arteries; 2nd ed,1974. Williams and Wilkins, Baltimore.

38. Anliker M, Casty M, Friedli P, Kubli R, Keller H. Noninvasive measurement of blood flow. In: Cardiovascular flow dynamics and measurements, 1977; Hwang NHC, Normann NA (eds). University Park Press, Baltimore.

39. Kornet L, Lambregts J, Hoeks APG, Reneman RS. Differences in near-wall shear rate in the carotid artery within subjects are associated with different intima-media thickness. Arterioscler Thromb Vasc Biol 1998; 18: 1877-84.

40. Alevriadou BR, McIntire LV. Rheology. In: Thrombosis and Hemorrhage 1994; Loscalzo J, Schafer AI (eds). Blackwell Science, Cambridge, MA.

41. Picker LJ, Warnock RA, Burns AR, Doerschuk CM, Berg EL, Butcher EC. The neutrophil selectin LECAM-1presents carbohydrate ligands to the vascular selectins ELAM-

1 and GMP-140. Cell 1991; 66: 921-33

42. Wells RE, Merrill EW, Gabelnick H. Shear-rate dependence of viscosity of blood: interaction of red cells and plasma proteins. Trans Soc Rheol 1962; 6: 19.

43. Motomiya M, Karino T. Flow patterns in the human carotid artery bifurcation. Stroke 1984; 15: 50-6.

44. Pritchard WF, Davies PF, Derafshi Z, Polacek DC, Tsao R, Dull RO, Jones SA, Giddens DP. Effects of wall shear stress and fluid recirculation on the localization of circulating monocytes in a three-dimensional flow model. J Biomechan 1995; 28: 1459-69.

45. Karino T, Goldsmith HL. Adhesion of human platelets to collagen on the walls distal to a tubular expansion. Microvasc Res 1979; 17: 238-62.

46. Rong JX, RangaswamyS, Shen L, Dave R, Chang YH, Peterson H, Hodis HN, Chisholm GM, Sevanian A. Arterial injury by cholesterol oxidation products causes endothelial dysfunction and arterial wall cholesterol accumulation. Arterioscler Thromb Vasc Biol 1998; 18: 1885-94.

47. Fry DL. Responses of the arterial wall to certain physical factors. In Atherogenesis: Initiating factors 1973. P. 93. Excerpta Medica, Amsterdam.

48. Stein PD, Sabbah HN, Anbe DH, Walburn FJ. Blood viscosity in the abdominal aorta and common iliac artery of man. Biorheol 1979; 16: 249-55.

49. Holme PA, Orvim U, Hamers MJAG, Solum NO, Brosstad FR, Barstad RM, Sakariassen KS. Shear-induced playtelet activation and platelet microparticle formation at blood flow conditions as in arteries with a severe stenosis. Arterioscler Thromb Vasc Biol 1997; 17: 646-53.

50. Davies PF. Flow-mediated endothelial mechanotransduction. [Review] Physiology Rev 1995; 75:519-60.

51. Diamond SL, Sharefkin JB, Dieffenbach C, Frasier-Scott K, McIntyre LV, Eskin SG. Tissue plasminogen activator messenger RNA levels increase in cultured human endothelial cells exposed to laminar shear stress. J Cell Physiol 1990; 143: 364-371.

52. Ranjan V, Xiao Z, Diamond SL. Constitutive nitric oxide synthase protein and mRNA levels are elevated in cultured human and bovine endothelial cells exposed to fluid shear stress. Am J Physiol 1995; 269: H550-55.

53. Galbusera M, Zoja C, Donadelli R, Paris S, Morigi M, Benigni A, Figliuzzi M, Remuzzi G, Remuzzi A. Fluid shear stress modulates von Willebrand factor release from human vascular endothelium. Blood 1997; 90: 1558-64.

54. Brown CH III, Leverett LB, Lewis CW, Alfrey CP Jr, Hellums JD. Morphological, biochemical and functional changes in human platelets subjected to shear stress. J Lab Clin Med 1975; 86:462-71.

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

56. Sakariassen KS, Aarts PA, de Groot PG, Houdijk WP, Sixma JJ. A perfusion chamber developed to investigate platelet interaction in flowing blood with human vessel wall cells, their extracellular matrix, and purified components. J Lab Clin Med 1983; 102; 522-35.

57. Van de Ven, TGM. Two colloidal particles subjected to an external field. In: Colloidal

Hydrodynamics, 1989; , pp. 384-91. Academic Press, London.

58. Xia A, Frojmovic MM. Aggregation efficiency of activated normal or fixed platelets in a simple shear field: Effect of shear and fibrinogen occupancy. Biophys J 1994; 66: 2190-2201.

59. Ruggeri ZM, Zimmerman TS. The complex multimeric composition of factor VIII/ von Willebrand factor. Blood 1981; 57: 1140-43.

60. Sporn LA, Chavin SI, Marder VJ, Wagner DD. Biosynthesis of von Willebrand protein by human megakaryocytes. J Clin Invest 1985; 76: 1102-6.

61. Jaffe EA, Hoyler LW, Nachman RL. Synthesis of antihemophilic factor antigen by cultured human endothelial cells. J Clin Invest 1973; 60: 914-21.

62. 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. Arterioscler 1986; 6: 24-33.

63. Sporn LA, Marder VJ, Wagner DD. Differing polarity of the constitutive and regulated pathways for von Willebrand factor in endothelial cells. J Cell Biol 1989; 108: 1283-9.

64. Ginsburg D, Handin RI, Bonthron DT, Donlon TA, Bruns GAP, Latt SA, Orkin SH. Human von Willebrand factor (vWF): Isolation of complementary DNA (cDNA) clones and chromosome localization. Science 1985: 228: 1401-6.

65. Sadler JE. Von Willebrand factor. J Biol Chem 1991; 266: 22777-80.

66. Sadler JE, Shelton-Inloes BB, Sorace JM, Harlan JM, Titani K, Davie EW. Cloning and characterization of two cDNA's coding for human von Willebrand factor. PNAS, USA 1985; 82: 6394-8.

67. Wagner DD, Lawrence SO, Ohlsson-Wilhelm BM, Fay PJ, Marder VJ. Topology and order of formation of interchain disulfide bonds in von Willebrand factor. Blood 1987; 69: 27-32.

68. Mayadas TN, Wagner DD. In vitro multimerization of von Willebrand factor is triggered by low pH. Importance of the propolypeptide and free sulfhydryls. J Biol Chem 1989; 264: 13497-503.

69. Titani K, Kumar S, Takio K, Ericsson LH, Wade RD, Ashida K, Walsh KA, Chopek MW, Sadler JE, Fujikawa K. Amino acid sequence of human von Willebrand factor. Biochem 1986; 25: 3171-84.

70. Lankhof H, van Hoeij M, Schiphorst ME, Bracke M, Wu YP, IJsseldijk MJ, Vink T, de Groot PG, Sixma JJ. A3 domain is essential for interaction of von Willebrand factor with collagen Type III. Thromb Haemost 1996; 75: 950-8.

71. Fujimura Y, Titani K. Structure and Function of von Willebrand factor. In: Thrombosis and Hemorrhage, 1994 ; Loscalzo J, Schafer AI (eds). Blackwell Scientific Publishing Co. Boston.

72. Ohmori K, Fretto LJ, Harrison RL, Switzer ME, McKee PA. Electron microscopy of human Factor VIII/von Willebrand effect of reducing reagents on structure and function. J Cell Biol 1982; 95: 632-40.

73. Slayter H, Loscalzo J, Bockenstedt, Handin RI. Native conformation of human von willebrand protein: analysis by electron microscopy and quasi-elastic light scattering. J Biol Chem 1985; 260: 8559-8563.

74. Beumer S, Heijnen HF, Ijsseldijk MJ, Orlando E, de Groot PG, Sixma JJ. Platelet

adhesion to fibronectin in flow: the importance of von Willebrand factor and glycoprotein Ib.Blood 1995; 86: 3452-60.

75. Siedlicki CA, Eppell SJ, Marchant RE. Interactions of human von Willebrand factor with a hydrophobic self-assembled monolayer studied by atomic force microscopy. J Biomed Mater Res 1994; 28: 971-80.

76. Siedlicki CA, Lestini BJ, Kottke-Marchant K, Eppell SJ, Wilson DL, Marchant RE. Shear-dependent changes in the three-dimensional structure of human von Willebrand factor. Blood 1996; 88: 2939-50.

77. Colombatti A, Bonaldo P. The superfamily of proteins with von Willebrand factor Type A-like domains: one theme common to components of extracellular matrix, hemostasis, cellular adhesion, and defense mechanisms. Blood 1991; 77: 2305-15.

78. Edwards YJK, Perkins SJ. The protein fold of the von Willebrand factor Type A domain is predicted to be similar to the open twisted ß-sheet flanked by α-helices found in human ras-p21. FEBS Lett 1995; 358: 283-6.

79. Emsley J, Cruz M, Handin R, Liddington R. Crystal structure of the von Willebrand factor A1 domain and implications for the binding of platelet glycoprotein Ib. J Biol Chem 1998; 273: 10396-401.

80. Celikel R, Varughese KI, Madhusudan, Yoshioka A, Ware J, Ruggeri ZM. Crystal structure of the von Willebrand factor A1 domain in complex with the function blocking NMC-4 fab. Nature Struct Biol 1998; 5: 189-94.

81. Jenkins PV, Pasi KJ, Perkins SJ. Molecular modeling of ligand and mutation sites of the type A domains of human von Willebrand factor and their relevance to von Willebrand's

disease. Blood 1998; 91: 2032-44.

82. Montgomery RR, Coller BS. Von Willebrand Disease. In Hemostasis and Thrombosis: basic principles and clinical practice. third edition, 1994; RW Colman, J. Hirsch VJ Marder, EW Salzman (eds). JB Lippincott Company, Philadelphia.

83. Sadler JE, Matsushita T, Dong Z, Tuley EA, Westfield LA. Molecular mechanism and classification of von Willebrand disease. Thromb Haemost 1995; 74: 161-66.

84. Tsai HM, Sussman II, Nagel RL. Shear stress enhances the proteolysis of von Willebrand factor in normal plasma. Blood 1994; 83: 2171-9.

85. Furlan M, Robles R, Lämmle B. Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. Blood 1996; 87: 4223-34.

86. Christophe O, Ribba AS, Baruch D, Obert B, Rouault C, Niinomi K, Piétu G, Meyer D, Girma JP. Influence of mutations and size of multimers in Type II von Willebrand disease upon the function of von Willebrand factor. Blood 1994; 83: 3553-61.

87. Blann A. von Willebrand factor and the endothelium in vascular disease. Br J Biomed Sci 1993; 50: 125-34.

88. Konstantopoulos K, Grotta JC, Sills C, Wu KK, Hellums JD. Shear-induced platelet aggregation in normal subjects and stroke patients. Thromb Haemost 1995; 74: 1329-34.

89. Nichols TC, Bellinger DA, Reddick RL, Read MS, Koch GG, Brinkhous KM, Griggs TR. Role of von Willebrand factor in arterial thrombosis: Studies in normal and von Willebrand disease pigs. Circulation 1991; 83[suppl IV]:IV-56-IV-64.

90. Federici AB, Mannucci PM, Fogato E, Ghidoni P, Matturri L. Autopsy findings in three

patients with von Willebrand disease Type IIB and Type III: presence of atherosclerotic lesions without occlusive arterial thrombi. Thromb Haemost 1993; 70: 758-61.

91. Hoylaerts MF, Nuyts K, Peerlinck K, Deckmyn H, Vermylen J. Promotion of binding of von Willebrand factor to platelet glycoprotein Ib by dimers of ristocetin. Biochem J 1995; 306: 453-63.

92. Andrews RK, Booth WJ, Gorman JJ, Castaldi PA, Berndt MC. Purification of botrocetin from Bothrops jararaca venom. Analysis of the botrocetin-mediated interaction between von Willebrand factor and the human platelet membrane glycoprotein Ib-IX complex. Biochemistry 1989; 28: 8317-26.

93. Gralnick HR, Coller BS. Platelets stimulated with thrombin and ADP bind von Willebrand factor to different sites than platelets stimulated with ristocetin. Clin Res 1983; 31: Abstr # 482A.

94. 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 GPIb in the initiation of shear-induced platelet aggregation. Thromb Haemost 1993; 69: 496-502.

95. Cranmer SL, Ulsemer P, Cooke BM, Yuan Y, Salem H, de la Salle C, Lanza F, Jackson SP, Berndt MC. The glycoprotein Ib/IX-vWF interaction mediates rolling and cytoskeletal reorganization in platelets and GPIb/IX transfected CHO cells. Blood 1998; 92: Abstr # 2888.

96. Savage B, Salvidar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. Cell 1996; 84: 289-97.

97. Savage B, Shattil SJ, Ruggeri ZM. Modulation of platelet function through adhesion receptors: A dual role for glycoprotein GPIIb-IIIa (integrin $\alpha_{IIb}\beta_3$) mediated by fibrinogen and glycoprotein Ib-von Willebrand factor. J Biol Chem 1992; 267: 11300-6.

98. Barabino GA, Wise RJ, Woodbury VA, Zhang B, Bridges KA, Hebbel HP, Lawler J, Ewenstein BM. Inhibition of sickle erythrocyte adhesion to immobilized thrombospondin by von Willebrand factor under dynamic flow conditions. Blood 1997; 89: 2560-7.

99. Lozcalzo J, Inbal A, Handin RI. Von Willebrand protein facilitates platelet incorporation in polymerizing fibrin. J Clin Invest 1986; 78: 1112-9.

100. Parker RI, Gralnick HR. Fibrin monomer induces binding of endogenous platelet von Willebrand factor to the glycocalicin portion of the platelet glycoprotein Ib. Blood 1987; 70: 1589-94.

101. Endenburg SC, Hantgan RR, Lindeboom-Blokzijl L, Lankhof H, Jerome WG, Lewis JC, Sixma JJ, de Groot PG. On the role of von willebrand factor in promoting platelet adhesion to fibrin in flowing blood. Blood 1995; 86: 4158-65.

102. Berliner S, Niiya K, Roberts JR, Houghten RA, Ruggeri ZM. Generation and characterization of peptide-specific antibodies that inhibit von Willebrand factor binding to glycoprotein IIb-IIIa without interacting with other adhesive molecules; Selectivity is conferred by Pro^{1743} and other amino acid residues adjacent to the sequence Arg^{1744}-Gly^{1745}-Asp^{1746}. J Biol Chem 1988; 263: 7500-5.

103. Miyata S, Goto S, Federici AB, Ware J, Ruggeri ZM. Conformational changes in the A1 domain of von Willebrand factor modulating the interaction with platelet glycoprotein Ibα. J Biol Chem 1996; 271:9046-53.

104. Piétu G, Ribba AS, Chérel G, Siguret V, Obert B, Rouault, Ginsberg D, Meyer D. Epitope mapping of inhibitory monoclonal antibodies to human von Willebrand factor by using recombinant cDNA libraries. Thromb Haemost 1994; 71: 788-92.

105. Matsushita T, Sadler JE. Identification of amino acid residues essential for von willebrand factor binding to platelet glycoprotein Ib; charged-to-alanine scanning mutagenesis of the A1 domain of human von Willebrand factor. J Biol Chem 1995; 270: 13406-414.

106. Cooney KA, Ginsburg D. Comparative analysis of Type 2b von Willebrand Disease mutations: Implications for the mechanism of von Willebrand factor binding to platelets. Blood 1996; 87: 2322-8.

107. Miyata S, Ruggeri ZM. Distinct attributes regulating von Willebrand factor A1 domain interaction with platelet glycoprotein Ibα under flow. J Biol Chem 1999; 274: 6586-93.

108. Furlan M, Stieger J, Beck EA. Exposure of platelet binding sites in von Willebrand factor by adsorption onto polystyrene latex particles. Biochim Biophys Acta 1987; 924: 27-37.

109. Sporn LA, Marder VJ, Wagner DD. Inducible secretion of large, biologically potent von Willebrand factor multimers. Cell 1986; 46:185-90.

110. Williams SB, McKeown LP, Krutzsch H, Hansmann K, Gralnick HR. Purification and characterization of human platelet von Willebrand factor. Br J Haematol 1994; 88: 582-91.

111. Parker RI, Gralnick HR. Identification of platelet glycoprotein GPIIb/IIIa as the major binding site for released platelet von-Willebrand factor. Blood 1986; 68: 732-6.

112. Parker RI, Gralnick HR. Inhibition of platelet-von Willebrand factor binding to platelets

by adhesion site peptides. Blood 1989; 74: 1226-30.

113. Castillo R, Monteagudo J, Escolar G, Ordinas A, Magallon M. Hemostatic effects of normal platelet transfusion in severe von Willebrand disease patients. Blood 1991; 77: 1901-5.

114. Sultan Y, Bouma BN, de Graaf S, Simeon J, Caen JP,Sixma JJ. Factor VIII related antigen in platelets of patients with von Willebrand's disease. Thromb Res 1977; 11: 23-30.

115. Fressinaud E, Baruch D, Rothschild C, Baumgartner HR, Meyer D. Platelet von Willebrand factor: evidence for its involvement in platelet adhesion to collagen. Blood 1987: 70: 1214-7.

116. Alevriadou BR, Moake JL, Turner NA, Ruggeri ZM, Folie BJ, Phillips MD, Schreiber AB, Hrinda ME, McIntyre LV. Real-time analysis of shear-dependent thrombus formation and its blockade by inhibitors of von Willebrand factor binding to platelets. Blood 1993; 81: 1263-76.

117. Hantgan RR, Francis CW, Marder VJ. Fibrinogen structure and physiology. In Hemostasis and Thrombosis:Basic priciples and clinial practice, third ed, 1994; Colman RW, Hirsh J, Marder VJ, Salzman EW (eds). JB Lippincott Co, Philadelphia.

118. Klinger MH, Kluter H. Immunocytochemical colocalization of adhesive proteins with clathrin in human blood platelets: further evidence for coated vesicle-mediated transport of von Willebrand factor, fibrinogen and fibronectin. Cell Tissue Res 1995; 270: 453-7.

119. Hatton MWC, Moar SL, Richardson M. Deendothelialization in vivo initiates a thrombogenic reaction at the rabbit aorta surface. Am J Pathol 1989; 135: 499-508.

120. Murray JC, Buetow KH, Donovan M, Hornung S, Motulsky AG, Disteche C, Dyer K,

Swisshelm K, Anderson J, Giblett E, Sadler E, Eddy R, Shows TB. Linkage dysequilibrium of plasminogen polymorphisms and assignment of the gene to human chromosome-6-Q26-6Q27. Am J Hum Gen 1987; 40: 338-50.

121. Wolfenstein-Todel C, Mossesson MW. Human plasma fibrinogen heterogeneity: evidence for an extended carboxyterminal sequence in a normal γ chain variant (γ'). Proc Natl Acad Sci UDSA 1980; 77: 5069-73.

122. Chung DW, Davie EW. γ and γ' chains of human fibrinogen are produced by alternative mRNA processing. Biochem 1984; 23: 4232-6.

123. Chen R, Doolittle RF. Identification of the polypeptide chains involved in the cross-linking of fibrin. Proc Natl Acad Sci USA 1969; 63: 420-7.

124. McKee PA, Mattock P, Hill RL. Subunit structure of human fibrinogen soluble fibrin, and cross-linked insoluble fibrin. Proc Natl Acad Sci USA 1970; 66: 738-44.

125. Schielen WJG, Adams HPHM, van Leuven K, Voskuilen M, Tesser GJ, Nieuwenhuizen W. The sequence gamma-(312-324) is a fibrin specific epitope. Blood 1991; 77: 2169-73.

126. Hocking CS, Laskowski M, Scheraga HA. Size and shape of bovine fibrinogen. J Am Chem Soc 1952; 74: 775.

127. Doolittle RF, Goldbaum DM, Doolittle LR. Designation of sequences involved in the "coiled-coil" interdominal connector in fibrinogen: construction of an atomic scale model. J Mol Biol 1978; 120: 311-25.

128. Marchant RE, Barb MD, Shainoff JR, Eppell SJ, Wilson DL, Siedlicki CA. Three dimensional structure of human fibrinogen under aqueous conditions visualized by atomic force microscopy. Thromb Haemost 1997; 77: 1048-51.

129. Taatjes DJ, Quinn AS, Jenny RJ, Hale P, Bovill EG, McDonagh J. Tertiary structure of the hepatic cell protein fibrinogen in fluid revealed by atomic force microscopy. Cell Biol Intnal 1997; 21: 1-12.

130. Nieuwenhuizen W, van Ruijven-Vermeer IAM, Nooijen WJ, Vermond A, Haverkate F, Hermans J. Recalculation of calcium-binding properties of human and rat fibrin(ogen) and their degradation products. Thromb Res 1981; 22: 653-7.

131. Nieuwenhuizen W, Haverkate F. Calcium-binding regions in fibrinogen. Ann NY Acad Sci 1983; 408: 92-6.

132. Kloczewiak M, Timmons S, Lukas TJ, Hawiger J. Platelet receptor recognition site on human fibrinogen. Synthesis and structure-function relationship of peptides corresponding to the carboxyterminal segment of the γ chain. Biochem 1984; 23: 1767-74.

133. Kloczewiak M, Timmons S, Bednarek A, Sakon M, Hawiger J. Platelet receptor recognition domain on the γ chain of human fibrinogen and its synthetic peptide analogs. Biochem 1989; 28: 2915–9.

134. Liu G, Matsueda G, Brown E, Frojmovic. The AGDV residues on the gamma chain carboxyl terminus of platelet-bound fibrinogen are needed for platelet aggregation. Biochim Biophys Acta 1997; 1343: 316-26.

135. Liu Q. Rooney MM. Kasirer-Friede A. Brown E. Lord ST. Frojmovic MM. Role of the gamma chain Ala-Gly-Asp-Val and Aalpha chain Arg-Gly-Asp-Ser sites of fibrinogen in coaggregation of platelets and fibrinogen-coated beads. Biochimica et Biophysica Acta. 1998;1385:33-42.

136. Rooney MM, Farrell DH, van Hemel BM, de Groot PG, Lord ST. The contribution of

the three hypothesized integrin-binding sites in fibrinogen to platelet-mediated clot retraction. Blood 1998; 92: 2374-81.

137. Kunicki TJ, Newman PJ, Amrani DL, Mossesson MW. Human platelet fibrinogen: purification and hemostatic properties. Blood 1985: 808-15.

138. Marguerie GA, Edgington TS, Plow EF. Interaction of fibrinogen with its platelet receptor as part of a multistep reaction in ADP-induced platelet aggregation. J Biol Chem 1980; 255: 154-61.

139. Peerschke EIB, Wainer JA. Examination of irreversible platelet-fibrinogen interactions. Am J Physiol 1985; 248: C466-72.

140. Bacon-Baguley T, Ogilvie ML, Gartner TK, Walz DA. Thrombospondin binding to specific sequences within the A-alpha- and B beta-chains of fibrinogen. J Biol Chem 1990; 265: 2317-23.

141. Liu Q, Frojmovic MM. Adhesion efficiency of unactivated and activated platelets to fibrinogen immobilized on polystyrene beads in a homogenous shear field. Submitted to Biophys J, April 1999.

142. Weber C, Springer TA. Neutrophil accumulation on activated, surface-adherent platelets in flow is mediated by interaction of Mac-1 with fibrinogen bound to alphaIIbbeta3 and stimulated by platelet-activating factor. J Clin Invest 1997; 100: 2085-93.

143. van de Stolpe A, Jacobs N, Hage WJ, Tertoolen L, van Kooyk Y, Novakova IR, de Witte T. Fibrinogen binding to ICAM-1 on EA.hy 926 endothelial cells is dependent on an intact cytoskeleton. Thromb Haemost 1996; 75: 182-9.

144. Legrand C, Dubernard V, Rabhi-Sabile V, Morandi DS. Functional and clinical

significance of thrombospondin. Platelets 1997; 8: 211-223.

145. Lawler J, Derick LH, Connoly JE, Chen JH, Chao FC. The structure of human thrombospondin. J Biol Chem 1985; 260: 3762-72.

146. Lawler J, Simons ER. Cooperative binding of calcium to thrombospondin. The effect of calcium on the circular dichroism and limited tryptic digestion of thrombospondin. J Biol Chem 1983; 258: 12098-101.

147. Agbanyo FR, Sixma JJ, de Groot PG, Languino LR, Plow EF. Thrombospondin-platelet interactions: Role of divalent cations, wall shear rate, and platelet membrane glycoproteins. J Clin Invest 1993; 92: 288-96.

148. Lawler J, Chao FC, Cohen CM. Evidence for calcium-sensitive structure in platelet thrombospondin; isolation and partial characterization of thrombospondin in the presence of calcium. J Biol Chem 1982; 257: 12257-65.

149. Karczewski J, Knudsen KA, Smith L, Murphy A, Rothman VL, Tuszynski GP. The interaction of thrombospondin with platelet glycoprotein $GPII_b$-III_a. J Biol Chem 1989; 264: 21322-26.

150. Asch AS, Leung LK, Polley MJ, Nachman RL. Platelet membrane topography: localization of thrombospondin and fibrinogen with the glycoprotein IIb-IIIa complex. Blood 1985; 66: 926-34.

151. Legrand C, Dubernard V, Kieffer N, Nurden AT. Use of a monoclonal antibody to measure the surface expression of thrombospondin following platelet activation. Eur J Biochem1988;171: 393-9.

152. Boukerche H, McGregor JL. Characterization of an anti-thrombospondin monoclonal

antibody (P8) that inhibits human blood platelet function. Normal binding of P8 to thrombin-activated Glanzmann thrombasthenic platelets. Eur J Biochem 1988; 171: 383-92.

153. Gao AG, Lindberg FP, Finn MB, Blystone SD, Brown EJ, Frazier WA. Integrin-associated protein is a receptor for the C-terminal domain of thrombospondin. J Biol Chem 1996; 271: 21-4.

154. Dorahy DJ, Thorne RF, Fecondos JV, Burns GF. Stimulation of platelet activation and aggregation by a carboxy-terminal peptide from thrombospondin binding to the integrin-associated protein receptor. J Biol Chem 1997; 272: 1323-30.

155. Bale MD. Noncovalent and covalent interactions of thrombospondin with polymerizing fibrin. Sem Thromb Hemos 1987; 13: 326-34.

156. Rabhile-Sabile S, Thibert V, Legrand C. Thrombospondin peptides inhibit the secretion-dependent phase of platelet aggregation. Blood Coag and Fibrinolys 1996; 7: 237-40.

157.Legrand C, Thibert V, Dubernard V, Begault B, Lawler J. Molecular requirements for the interaction of thrombospondin with thrombin-activated platelets: modulation of platelet aggregation. Blood 1992; 79: 1995-2003.

158. Fukimoto T, Fujimura K, Noda M, Takafuta T, Shimomura T, Kuramoto A. 50kDa integrin-associated protein does not detectably influence several functions of GPIIb-IIIa complex in human platelets. Blood 1995; 86: 2174-82.

159. Beumer S, Ijsseldijk MJ, de Groot PG, Sixma JJ. Platelet adhesion to fibronectin in flow: dependence on surface concentration and shear rate, role of platelet membrane glycoprotein GP IIb/IIIa and VLA-5, and inhibition by heparin. Blood 1994; 84: 11: 3724-33.

160. Ohno M, Ohno N, Kefalides NA. Studies on human laminin and laminin-collagen complexes. Connect Tissue Res 1991; 25; 251-63.

161. Chen CH, Clegg DO, Hansma HG. Structures and dynamic motion of laminin-1 as observed by atomic force microscopy. Biochem 1998; 37: 8262-67.

162. Hindriks G, IJsseldijk MJ, Sonnenberg A, Sixma JJ, de Groot PG. Platelet adhesion to laminin: role of Ca 2+ and Mg 2+ ions, shear rate, and platelet membrane glycoproteins. Blood 1992; 79: 928-35.

163. Mills DCB. ADP receptors on platelets. Thromb Haemost 1996; 76: 835-56.

164. Born GV. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 1962; 194:927-9.

165. Hellem AJ, Borchgrevink CF, Ames SB. The role of red cells in haemostasis: the relation between hematocrit, bleeding time and platelet adhesiveness. Br J Haematol 1961;7: 43.

166. Sharis PJ, Cannon CP, Loscalzo J. The antiplatelet effects of ticlopidine and clopidogrel. Ann Internal Med 1998; 129: 394-405.

167. Yao SK, Ober JC, McNatt J, Benedict CR, Rosolowsky M, Anderson HV, Cui K, Maffrand JP, Campbell WB, Buja LM, Willerson JT. ADP plays an important role in mediating platelet aggregation and cyclic flow variations in vivo in stenosed and endothelium-injured canine coronary arteries. Circulation Res 1992; 70: 39-48.

168. Oda A, Yokoyama K, Murata M, Tokuhira M, Nakamura K, Handa M, Watanabe K, Ikeda Y. Protein tyrosine phosphorylation in human platelets during shear stress-induced platelet aggregation (SIPA) is regulated by glycoprotein (GP) Ib/IX as well as GPIIb/IIIa and

requires intact cytoskeleton and endogenous ADP. Thromb Haemost 1995; 74: 736-42.

169. Ashby B. Nucleotide metabolism. In: Platelet responses and metabolism 1987; . Holmsen H (ed). CRC Press, Boca Raton.

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

171. Akkerman JWN, Gorter G, Schrama L, Holmsen H. A novel technique for rapid determination of energy consumption in platelets: determination of different energy consumption associated with three secretory responses. Biochem J 1983; 210: 145-55.

172. Editorial. The bleeding-time and the hematocrit. Lancet 1984; 1: 997-1002.

173. Jantzen HM, Gousset L, Bhaskar V, Vincent D, Tai A, Reynolds EE, Conley PB. Evidence for two distinct G-protein-coupled ADP receptors mediating platelet activation. Thromb Haemost 1999; 81: 111-7.

174. Kunapuli SP, Dangelmaier C, Jin J, Kim YB, Daniel JL, Rao AK. Role of intracellular signaling events in ADP-induced platelet aggregation. Blood Suppl I 1998; 92: Abstr #103.

175. Fox JEB, Shattil SJ, Kinlough-Rathbone RL, Richardson M, Packham MA, Sanan DA. The platelet cytoskeleton stabilizes the interaction between $\alpha_{IIb}\beta_3$ and its ligand and induces selective movements of ligand-occupied integrin. J Biol Chem 1996; 7004-11.

176. May JA, Heptinstall S, Spangenberg P. Changes in the composition of the platelet cytoskeleton in response to ADP: effects of MK-852 and ARL 66096. Blood Coag and Fibrinolysis 1996; 7: 221-4.

177. Vanags DM, Lloyd JV, Rodgers SE, Bochner F. ADP, adrenaline and serotonin stimulate inositol 1,4,5-triphosphate production in human platelets. Eur J Pharmacol 1998;

358: 93-100.

178. Gachet C, Calvete JP. ADP induced blood platelet activation : a review. Nouv Rev Fr Hematol 1991; 33: 347-58.

179. Packham MA, Livne AA, Ruben DH, Rand ML. Activation of phospholipase C and protein kinase C has little involvement in ADP-induced primary aggregation of human platelets: effects of diacylglycerols, the diacylglycerol kinase inhibitor R59022, staurosporine and okadaic acid. Biochem J 1993; 849-56.

180. Mann KG. Prothrombin and thrombin. In Hemosatsis and Thrombosis: basic principles and practice, third ed 1994; eds Colman RW, Hirsh J, Marder VJ, Salzman EW. JB Lippincott Co, Philadelphia: 184-199.

181. Stubbs MT, Bode W. A player of many parts: the spotlight falls on thrombin's structure. Thromb Res 1993; 69: 1-58.

182. Bevers EM, Comfurius P, Zwaal RF. Changes in membrane phospholipid distribution during platelet activation. Biochim Biophys Acta 1983; 736: 57-66.

183. Ravanat C, Archipoff G, Beretz A, Freund G, Cazenave JP, Freyssinet JM. Use of annexin-V to demonstrate the role of phosphatidyl serine exposure in the maintenance of hemostatic balance by endothelial cells. Biochem J 1992: 282: 7-13.

184. Robinson RA, Worfolk L, Tracy PB. Endotoxin enhances the expression of monocyte prothrombinase activity 1992; 79: 406-16.

185. Béguin S and Kumar R. Thrombin, fibrin and platelets: a resonance loop in which von Willebrand factor is a necessary link. Thromb Haemost 1997; 78: 590-4.

186. Colwell NS, Blinder MA, Tsiang M, Gibbs CS, Bock PE. Allosteric effects of a

monoclonal antibody against thrombin exosite II. Biochem 1998; 37: 15057-65.

187. Mathews II, Padmanabhan KP, Ganesh V, Tulinsky A. Crystallographic structures of thrombin complexed with thrombin receptor peptides: existence of expected and novel binding modes. Biochem 1994; 33: 3266-79.

188. Olson ST, Björk I, Shore JD. Kinetic characterization of heparin-catalyzed and uncatalyzed inhibition of blood coagulation proteases by antithrombin. In: Methods in Enzymol 1993; 222: 525-60.

189. Ye J, Esmon CT, Johnson AE. The chondroitin sulfate moiety of thrombomodulin binds a second molecule of thrombin. J Biol Chem 1993; 268: 2373-9.

190. Arni RK, Padmanabhan KP, Wu TP, Tulinsky A. Structures of the noncovalent complexes of human and bovine prothrombin fragment 2 with human PPACK-thrombin. Biochem 1993; 32: 4727-37.

191. De Cristofaro R, de Candia E, Croce G, Morosetti R, Landolfi R. Binding of human α-thrombin to platelet GPIb: energetics and functional effects. Biochem J 1998; 332: 643-50.

192. Ternisien C, Jandrot-Perrus M, Huisse MG, Guillin MC. Effect of phosphopyridoxylation on thrombin interaction with platelet glycoprotein Ib. Blood Coag Fibrinolys 1991; 2: 521-8.

193. Jackman MP, Parry MA, Hofsteenge J, Stone SR. Intrinsic fluorescence changes and rapid kinetics of the reaction of thrombin with hirudin. J Biol Chem 1992; 267: 15375-83.

194. Hogg PJ, Jackson CM, Labonowski JK, Bock PE. Binding of fibrin monomer and heparin to thrombin in a ternary complex alters the environment of the thrombin catalytic site, reduces affinity for hirudin, and inhibits cleavage of fibrinogen. J Biol Chem 1996; 271:

26088-95.

195. Fredenburgh JC, Stafford AR, Weitz JI. Evidence for allosteric linkage between exosites 1 and 2 of thrombin. J Biol Chem 1997; 272: 25493-9.

196. Vu TKH, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 1991; 64: 1057-68.

197. Kahn ML.Zheng YW, Huang W, Bigornia V, Zeng D, Moff S, Farese RV Jr, Tam C, Coughlin SR. A dual thrombin receptor system for platelet activation. Nature1998; 394:690-4.

198. Greco NJ, Jones GD, Tandon NN, Kornhauser R, Jackson B, Jamieson GA. Differentiation of the two forms of GPIb functioning as receptors for alpha-thrombin and von Willebrand factor: Ca^{2+} responses of protease-treated human platelets activated with alpha thrombin and the tethered ligand peptide. Biochem 1996; 35: 915-21.

199. Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. J Clin Invest 1999; 103: 879-87.

200. Hayes KL, Tracy PB. The platelet high affinity binding site for thrombin mimcs hirudin, modulates thrombin-induced platelet activation, and is distinct from the glycoprotein Ib-IX-V complex. J Biol Chem 1999; 274: 972-80.

201. Jamieson GA. Pathophysiology of platelet thrombin receptors. Thromb Haemost 1997; 78: 242-6.

202. Mazzucato M, De Marco L, Masotti A, Pradella P, Bahou WF, Ruggeri ZM. Characterization of the initial α-thrombin interaction with glycoprotein Ibα in relation to

platelet activation. J Biol Chem 1998; 273: 1880-1887.

203. McKeown LP, Williams SB, Hansmann KE, Krutzsch, Gralnick HR. Glycoprotein Ibα peptides inhibit thrombin and SFLLRN-induced platelet aggregation. J Lab Clin Med 1996; 128: 492-5.

204. Brass LF, Vassallo RR, Belmonte E, Ahuja M, Cichowski K, Hoxie JA. Structure and function of the human platelet thrombin receptor: studies using monoclonal antibodies against a defined epitope within the receptor N-terminus. J Biol Chem 1992; 267: 13795-8.

205. McNicol A, Sutherland M, Zou R, Drouin J. Defective thrombin-induced calcium changes and aggregation of Bernard-Soulier platelets are not associated with deficient moderate-affinity receptors. Arterioscler Thromb Vasc Biol 1996; 16: 628-32.

206. Darrow AL, Fung-Leung WP, Ye RD, Santulli RJ, Cheung WM, Deriean CK, Burns CL, Damiano BP, Zhou L, Keenan CM, Peterson PA, Andrade-Gordon P. Biological consequences of thrombin receptor deficiency in mice. Thromb Haemost 1996; 76: 860-6.

207. Michelson AD, Ellis PA, Barnard MR, Matic GB, Viles AF, Kestin AS. Downregulation of the platelet surface glycoprotein Ib-IX complex in whole blood stimulated by thrombin, adenosine diphosphate, or an in vivo wound. Blood 1991; 77: 770-9.

208. Rao GHR, Peller JD, White JG. Influence of ionized calcium on thrombin-induced downregulation of GP Ib-IX receptors on human platelets. Thromb Res 1997; 85: 23-31.

209. Brass LF, Molino M. Protease-activated G protein-coupled receptors on human platelets and endothelial cells. Thromb Haemost 1997; 78: 234-41.

210. Giesberts AN, van Willigen G, Lapetina EG, Akkerman JN. Regulation of platelet glycoprotein IIb/IIIa (integrin $\alpha_{IIB}\beta_3$) function via the thrombin receptor. Biochem J 1995;

309: 613-20.

211. Bryan RMJr. Cerebral blood flow and energy metabolism during stress. Am J Physiol 1990; 259: H269-80.

212. Muller JE, Stone PH, Turi ZG, Rutherford JD, Czeiler CA, Parker C, Poole WK, Passamani E, Roberts R, Robertson T, Sobel BE, Willerson JT, Braunwald E, the Milis Study Group. Circadian variation in the frequency of onset of acute myocardial infarction. N Engl J Med 1985; 313: 1315-22.

213. Tofler GH, Brezinski D, Schafer AI, Czeisler CA, Rutherford JD, Willich SN, Gleason RE, Williams GH, Muller JE. Concurrent morning increase in aggregability and the risk of myocardial infarction and sudden cardiac death. N Engl J Med 1987; 316: 1514-8.

214. Gilman AG. Guanine nucleotide binding regulatory proteins and dual conrol of adenylate cyclase. J Clin Invest 1984; 73: 1-4.

215. Grant JA, Scrutton MC. Novel a2-adrenoreceptors primarily responsible for inducing human platelet aggregation. Nature 1979; 277: 659-61.

216. MacFarlane DE, Wright BL, Stump DC. Use of (methyl ^{3}-H) yohimbine as a radiolabeled ligand for alpha-2 adrenoreceptors on intact platelets. Comparison with dihydroergocryptine. Thromb Res 1981; 24: 31-43.

217. Plow EF, Marguerie GA. Induction of fibrinogen receptor on human platelets by epinephrine and ADP. J Biol Chem 1980: 255: 10971-7.

218. Goto S, Ikeda Y, Murata M, Handa M, Takahashi E, Yoshioka A, Fujimura Y, Fukuyama M, Handa S, Ogawa S. Epinephrine augments shear-induced platelet aggregation. Circulation 1992; 86: 1859-63.

219. Lanza F, Beretz A, Stierlé A, Hanau D, Kubina M, Cazenave JP. Epinephrine potentiates human platelet activation but is not an aggregating agent. Am J Physiol (Heart Circ Physiol) 1988; 255: H1276-88.

220. Nakamura T, Ariyoshi H, Kambayashi J, Ikeda M, Kawasaki T, Sakon M, Monden M. Effect of low concentration of epinephrine on human platelet aggregation analyzed by particle counting method and confocal microscopy. Comment in: J Lab Clin Med 1997; 130: 262-70.

221. Spalding A, Vaitkevicius H, Dill S, MacKenzie S, Schmaier A, Lockette W. Mechanism of epinephrine-induced platelet aggregation. Hypertension 1988; 31: 603-7.

222. Wang X, Yanagi S, Yang C, Inatome R, Yamamura H. Tyrosine phosphorylation and Syk activation are involved in thrombin-induced aggregation of epinephrine-potentiated platelets. J Biochem 1997; 121: 325-30.

223. Baldassare D, Mores N, Colli S, Pazzucconi F, Sirtori CR, Tremoli E. Platelet alpha 2-adrenergic receptors in hypercholesterolemia: a relationship between binding studies and epinephrine-induced platelet aggregation. Clin Pharmacol Therapeut 1997; 61: 684-91.

224. Hamberg M, Svensson J, Samuelsson B. Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. Proc Natl Acad Sci 1975; 72: 2994-8.

225. MacIntyre DE, Armstrong RA. Agonists and receptors: prostaglandins and thromboxanes. In: Platelet responses and Metabolism, 1987, ed. Holmsen H. Boca Raton CRC Press, V. II: 93.

226. Marcus AJ. The role of lipids in platelet function: with particular reference to the arachidonic acid pathway. J Lipid Res 1978; 19: 793-84.

227. Weiss HJ, Willis AL, Kuhn D, Brand H. Prostaglandin E_2 potentiation of platelet aggregation induced by LASS endoperoxide: absent in storage pool disease, normal after aspirin ingestion. Br J Haematol 1976; 32: 257-72.

228. Willerson JT, Golino P, Eidt J, Campbell WB, Buja LM. Specific platelet mediators and unstable coronary lesions. Experimental evidence and potential clinical implications. Circulation 1989; 80: 198-20.

229. Ware J. Molecular analyses of the platelet glycoprotein Ib-IX-V receptor. Thromb Haemost 1998; 79: 466-78.

230. Clemetson KJ. Platelet GPIb-V-IX complex. Thromb Haemost 1997; 78: 266-70.

231. Fox JE. Linkage of a membrane skeleton to integral membrane glycoproteins in human platelets; identification of one of the glycoproteins as glycoprotein Ib. J Clin Invest 1985; 76: 1673-83.

232. Andrews RK, Fox JE. Identification of a region in the cytoplasmic domain of the platelet membrane glycoprotein Ib-IX complex that binds to purified actin-binding protein. J Biol Chem 1992; 267: 18605-11.

233. Du X, Harris SJ, Tetaz TJ, Ginsberg MH, Berndt MC. Association of a phospholipase A2 (14-3-3) protein with the platelet glycoprotein Ib-IX complex. J Biol Chem 1994; 269: 18287-90.

234. Du X, Fox JE, Pei S. Identification of a binding sequence for the 14-3-3 protein within the cytoplasmic domain of the adhesion receptor, platelet glycoprotein Ib alpha. J Biol Chem 1996; 271: 7362-7.

235. Calverley DC, Kavanagh TJ, Roth GJ. Human signaling protein 14-3-3zeta interacts

with platelet glycoprotein Ib subunits Ibalpha and Ib beta. Blood 1998; 91: 1295-303.

236. Kobe B, Deisenhofer J. Crystal structure of porcine ribonuclease inhibitor, a protein with leucine-rich repeats. Nature 1993; 366: 751-6.

237. Fox JE, Aggerbeck LP, Berndt MC. Structure of the glycoprotein Ib.IX complex from platelet membranes. J Biol Chem 1988; 4882-90.

238. Judson PA, Antsee DJ, Clamp JR. Isolation and characterization of the major oligosaccharide of human platelet glycoprotein GPIb. Biochem J. 1982; 205: 81-90.

239. Okumura T, Jamieson GA. Platelet glycocalicin. I. Orientation of glycoproteins of the human platelet surface. J Biol Chem 1976_a; 251: 5944-9.

240. Okumura T, Jamieson GA. Platelet glycocalicin II. Purification and characterization. J Biol Chem 1976_b; 251: 5950-5.

241. Coller BS, Kalomiris E, Steinberg M, Scudder LE. Evidence that glycocalicin circulates in normal plasma. J Clin Invest 1984; 73: 794-9.

242. Lopez JA, Leung B, Reynolds CC, Li CQ, Fox JEB. Efficient plasma membrane expression of a functional platelet glycoprotein Ib-IX complex requires the presence of its three subunits. J Biol Chem 1992; 267: 12851-9.

243. Meyer S, Kresbach G, Haring P, Schumpp-Vonach B, Clemetson KJ, Hadvary P, Steiner B. Expression and characterization of functionally active fragments of the platelet glycoprotein (GP) Ib-IX complex in mammmalian cells. Incorporation of GPIb alpha into the cell surface membrane. J Biol Chem 1993; 268: 20555-62.

244. Peterson EJ, Posthumus E, Sixma JJ. Functional expression of single chain glycoprotein Ib alpha on the surface of COS cells and BHK cells. Thromb Haemost 1996; 76: 768-73.

245. Berger G, Massé JM, Cramer EM. Alpha-granule membrane mirrors the platelet plasma membrane and contains the glycoproteins Ib, IX and V. Blood 1996; 87:1385-95.

246. Asch AS, Adelman B, Fujimoto M, Nachman RL. Identification and isolation of a platelet GPIb-like protein in human umbilical vein endothelial cells and bovine aortic smooth muscle cells. J Clin Invest 1988; 81: 1600-7.

247. Sprandio JD, Shapiro SS, Thiagarajan P, McCord S. Cultured human umbilical vein endothelial cells contain a membrane glycoprotein immunologically related to platelet glycoprotein Ib. Blood 1988; 71: 243-7.

248. Wu G, Essex DW, Meloni FJ, Takafuta T, Fujimura K, Konkle BA, Shapiro SS. Human endothelial cells in culture and in vivo express on their surface all four components of the glycoprotein Ib/IX/V complex. Blood 1997; 90: 2660-9.

249. Perrault C, Lankhof H, Pidard D, Kerbiriou-Nabias D, Sixma JJ, Meyer D, Baruch D. Relative importance of the glycoprotein Ib-binding domain and the RGD sequence of von Willebrand factor for its interaction with endothelial cells. Blood 1997; 90: 2335-44.

250. Michelson AD, Benoit SE, Furman MI, Barnard MR, Nurden P, Nurden AT. The platelet surface expression of glycoprotein V is regulated by two independent mechanisms: proteolysis and a reversible cytoskeletal-mediated redistribution to the surface-connected canalicular system. Blood 1996; 87: 1396-1408.

251. LaRosa CA, Rohrer MJ, Benoit SE, Barnard MR, Michelson AD. Neutrophil cathepsin G modulates the platelet surface expression of the glycoprotein (GP) Ib-IX complex by proteolysis of the von Willebrand factor binding site on GPIbα and by a cytoskeletal-mediated redistribution of the remainder of the complex. Blood 1994; 84:158-168.

252. Budarf ML, Konkle BA, Ludlow LB, MICHAUD D, Li M, Yamashiro DJ, McDonald-McGinn D, Zackai EH, Driscoll DA. Identification of a patient with Bernard-Soulier syndrome and a deletion in the DiGeorge/velocardio-facial chromosomal region in 22q11.2. Hum Mol Genet 1995; 3: 763-6.

253. George JN, Nurden AT. Inherited disorders of the platelet membrane: Glanzmann thrombasthenia, Bernard-Soulier syndrome, and other disorders.In Hemostasis and Thrombosis: Basic principles and practice, third ed 1994; Colman RW, Hirsh J, Marder VJ, Salzman EW (eds). JB Lippincott Co, Philadelphia.

254. Ware J, Russell SR, Marchese P, Murata M, Mazzucato M, De Marco L, Ruggeri ZM. Point mutation in a leucine-rich repeat of platelet glycoprotein Ibα resulting in the Bernard-Soulier syndrome. J Clin Invest 1993; 92: 1213-20.

255. Miller JL. Platelet-type von Willebrand disease. Thromb Haemost 1996; 75: 865-9.

256. 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.

257. Frederickson BJ, Dong JF, McIntire LV, Lopez JA. Shear-dependent rolling on von Willebrand factor of mammalian cells expressing the platelet glycoprotein Ib-IX-V complex. Blood 1998; 92: 3684-93.

258. De Marco L, Mazzucato M, Masotti A, Fenton JW, Ruggeri ZM. Function of glycoprotein Ibα in platelet activation induced by α-thrombin. J Biol Chem 1991; 266: 23776- 83.

259. Dong JF, Sae-Tung G, Lopez JA. Role of glycoprotein V in the Formation of the

platelet high-affinity thrombin-binding site. Blood 1997; 89: 4355-63.

260. Jandrot-Perrs M, Bouton MC, Lanza F, Guillin MC. Thrombin interactions with platelet membrane glycoprotein Ib. Sem Thromb Haemost 1996; 22: 151-6.

261. Yamamoto N, Kitawaga H, Tanoue K, Yamazaki H. Monoclonal antibody to glycoprotein Ib inhibits both thrombin- and ristocetin-induced platelet aggregations. Thromb Res 1985; 39: 751-9.

262. Coller BS, Peerschke EI, Scudder LE, Sullivan CA. Studies with a murine monoclonal antibody that abolishes ristocetin-induced binding of von Willebrand factor to platelets; additional evidence in support of GPIb as a platelet receptor for von Willebrand factor. Blood 1983; 61: 99-110.

263. De Marco L, Mazzucato M, Masotti A, Ruggeri ZM. Localization and characterization of an α-thrombin-binding site on platelet glycoprotein Ibα. J Biol Chem 1994; 269: 6478-84.

264. Peng M, Lu W, Kirby EP. Characterization of three alboaggregins purified from Trimeresurus albolabris venom. Thromb Haemost 1992; 67: 702-7.

265. Peng M, Lu W, Beviglia L, Niewiarowski S, Kirby EP. Echicetin: a snake venom protein that inhibits binding of von Willebrand factor and alboaggregins to platelet glycoprotein Ib. Blood 1993; 81: 2321-8.

266. Peng M, Emig FA, Mao A, Lu W, Kirby EP, Niewiarowski S, Kowalska MA. Interaction of echicetin with a high affinity thrombin binding site on platelet glycoprotein GPIß. Thromb Haemost 1995; 74: 954-7.

267. Berndt MC, Romo GA, Kenny DA, López, Andrews RK. Identification of monoclonal antibody epitopes and the ristocetin-dependent binding site on the α-chain of the platelet

GPIb-IX-V complex using canine-human chimaeras. Blood Suppl 1998; 92:Abstr # 2890.

268. Du X, Ginsberg MH. Integrin $\alpha_{IIb}\beta_3$ and platelet function. Thromb Haemost 1997; 96-100.

269. Gillis S, Furie BC, Furie B. Interactions of neutrophils and coagulation proteins. Sem Hematol 1997; 336-42.

270. Woods VL Jr, Wolff LE, Keller DM. Resting platelets contain a substantial centrally-located pool of GPIIb-IIIa complex which may be accessible to some but not other extracellular proteins. J Biol Chem 1986; 261: 15242-51.

271. Weisel JW, Nagaswami C, Vilaire G, Bennett JS. Examination of the platelet membrane glycoprotein Iib-IIIa complex and its interaction with fibrinogen and other ligands by electron microscopy. J Biol Chem 1992; 267: 16637-43.

272. Rivas GA, Aznarez JA, Usobiaga P, Saiz JL, Gonzalez-Rodriguez J. Molecular characterization of the human platelet integrin GPIIb/IIIa and its constituent glycoproteins. Eur Biophys J 1991; 19: 335-45.

273. Lam SCT. Isolation and characterization of a chymotryptic fragment of platelet glycoprotein IIb-IIIa retaining arg-gly-asp binding activity. J Biol Chem 1992; 267: 5649-55

274. Haas TA, Plow EF. The cytoplasmic domain of $\alpha_{IIb}\beta_3$. J Biol Chem 1996; 271: 6017-26.

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

276. Sims PJ, Ginsberg MH, Plow EF, Shattil SJ. Effect of platelet activation on the conformation of the plasma membrane glycoprotein IIb-IIIa complex. J Biol Chem 1991;

266: 7345-52.

277. Nurden P, Humbert M, Piotrowicz RS, Bihour C, Poujol C, Nurden AT, Kunicki TJ. Distribution of ligand-occupied $\alpha_{IIb}\beta_3$ in resting and activated human platelets determined by expression of a novel class of ligand-induced binding site recognized by monoclonal antibody AP-6. Blood 1996; 88: 887-99.

278. Tomiyama Y, Tsubakio T, Piotrowicz RS, Kurata Y, Kunicki TJ. The arg-gly-asp (RGD) recognition site of platelet glycoprotein IIb-IIIa on nonactivated platelets is accessible to high-affinity macromolecules. Blood 1992;79: 2303-12.

279. Beer JH, Springer K, Coller BS. Immobilized arg-gly-asp (RGD) peptides of varying lengths as structural probes of the platelet glycoprotein Iib/IIIa receptor. Blood 1992; 117-28.

280. D'Souza SE, Ginsburg MH, Matsueda GR, Plow EF. A discrete sequence in a platelet integrin is involved in ligand recognition. Nature 1991; 350: 66-8.

281. 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.

282. Charo IF, Nannizzi L, Phillips DR, Hsu MA, Scarborough RM. Inhibition of fibrinogen binding to GPIIb-IIIa by a GPIIIa peptide. J Biol Chem 1991; 266: 1414-21.

283. Lam SC, Plow EF, Smith MA, Andrieux A, Ryckwaert JJ, Marguerie G, Ginsberg MH. Evidence that arginyl-glycyl-aspartate peptides and fibrinogen gamma chain peptides share a common binding site on platelets. J Biol Chem 1987; 262: 947-50.

284. Hu DD, White CA, Panzer-Knodle S, Page JD, Nicholson N, Smith JW. A new model of dual interacting ligand binding sites on integrin $\alpha_{IIb}\beta_3$. J Biol Chem 1999; 274: 4633-9.

285. Hillery CA, Smyth SS, Parise LV. Phosphorylation of human platelet glycoprotein IIIa

(GPIIIa). J Biol Chem 1991; 266: 4663-9.

286. Chen YP, Djaffar I, Pidard D, Steiner B, Cietur AM, Caen JP, Rosa JP. Ser 752 to Pro 752 mutation in the cytoplasmic domain of integrin $ß_3$ subunit and defective activation of platelet integrin $\alpha_{IIb}ß_3$ (GPIIb-IIIa) in a variant of Glanzmann's thrombasthenia. Proc Natl Acad Sci USA 1992; 89: 10169-73.

287. Kashiwagi H, Schwartz MA, Eigenthaler M, Davis KA, Ginsberg MH. Affinity modulation of platelet integrin $\alpha_{IIb}ß_3$ by $ß_3$-endonexin, a selective binding partner of the $ß_3$ integrin cytoplasmic tail. J Cell Biol 1997; 137: 1433-1443.

288. Hughes PE, Diaz-Gonzales F, Leong L, Wu C, McDonald JA, Shattil SJ, Ginsberg MH. Breaking the integrin hinge; a defined structural constraint regulates integrin signaling. J Biol Chem 1996; 271: 6571-4.

289. Shiraga M, Tomiyama Y, Honda S, Suzuki H, Kosugi S, Tadokoro S, Kanakura Y, Tanoue K, Kurata Y, Matsuzawa Y. Involvement of the Na^+/Ca^{2+} exchanger in inside-out signaling through the platelet integrin αIIbß3. Blood 1998; 92: 3710-20.

290. Honda S, Tomiyama Y, Aoki T, Shiraga M, Kurata Y, Seki J, Matsuzawa Y. Association between ligand-induced conformational changes of integrin $\alpha_{IIb}ß_3$ and $\alpha_{IIb}ß_3$-mediated intracellular signaling. Blood 1998; 92: 3675-83.

291. Tokohira M, Handa M, Kamata T, Oda A, Katayama M, Tomiyama Y, Murata M, Kawai Y, Watanabe K, Ikeda Y. A novel regulatory epitope defined by a murine monoclonal antibody to the platelet GPIIb-IIIa complex ($\alpha_{IIb}ß_3$ integrin). Thromb Haemost 1996; 76: 1038-46.

292. Fox JEB, Lipfert L, Clark EA, Reynolds CC, Austin CD, Brugge JS. On the role of the

platelet membrane skeleton in mediating signal transduction. Association of GPIIb-IIIa pp60c-src, pp62c-yes, and the p21-ras GTPase-activating protein with the membrane skeleton. J Biol Chem 1993; 268: 25973-84.

293. Schootemeijer A, van Willigen G, van der Vuurst H, Tertoolen LGJ, De Laat SW, Akkerman JWN. Lateral mobility of integrin $\alpha_{IIb}\beta_3$ (Glycoprotein IIb/IIIa) in the plasma membrane of a human megakaryocyte. Thromb Haemost 1997; 77: 143-9.

294. Fox JEB, Shattil SJ, Kinlough-Rathbone RL, Richardson M, Packham MA, Sanan DA. The platelet cytoskeleton stabilizes the interaction between $\alpha_{IIB}\beta_3$ and its ligand and induces the selective movements of ligand-occupied integrin.J Biol Chem 1996; 271: 7004-11.

295. Erb EM, Tangemann K, Bohrmann, Müller B, Engel J.Integrin $\alpha_{IIB}\beta_3$ reconstituted into lipid bilayers is nonclustered in its activated state but clusters after fibrinogen binding. Biochem 1997; 36: 7395-402.

296. Golden A, Brugge JS, Shattil SJ. Role of platelet membrane glycoprotein Iib-IIIa in agonist-induced tyrosine phosphorylation of platelet proteins. J Cell Biol 1990; 111: 3117-27.

297. Lipfert L, Haimovich B, Schaller MD, Cobb BS, Parsons JT, Brugge JS. Integrin-dependent phosphorylation and activation of the tyrosine kinase pp125FAK in platelets. J Cell Biol 1992; 119: 905-12.

298. Fox JEB, Taylor RG, Taffarel M, Boyles JK, Goll DE. Evidence that activation of platelet calpain is induced as a consequence of binding of adhesive ligand to the integrin, glycoprotein Iib-IIIa. J Cell Biol 1993; 120: 1501-7.

299. Jackson SP, Schoenwaelder SM, Yuan Y, Salem HH, Cooray P. Non-receptor protein

tyrosine kinases and phosphatases in human platelets. Thromb Haemost 1996; 76: 640-50.

300. O'Toole TE, Ylanne J, Culley BM. Regulation of affinity states through an NPXY motif in the ß subunit cytoplasmic domain. J Biol Chem 1995; 270: 8553-8.

301. Wencel-Drake et al, Co-immunoprecipitation of AP-2-adaptor proteins and integrin $\alpha_{IIb}ß_3$. Blood 1998; 92: Abstr # 1415.

Introduction to Chapter 2

A previous study by Dr. Goldsmith et al indicated that in the absence of exogenous soluble ligands, the aggregation of platelets activated with low concentrations of ADP (0.7 μM), in tube flow, was shear-rate dependent and mediated by a surface-expressed protein other than fibrinogen. In this study, we investigated the eventuality that von Willebrand factor was a candidate for mediating this aggregation. The participation by the glycoprotein receptors Ib and IIb/IIIa was also assessed. The results of this study were published in Thrombosis and Hemostasis 1997; 77: 568-76, and are presented in Appendix 1 following the main body of the thesis*.

* Please note. I have chosen to add this section as an appendix since my contribution to this manuscript was partial, and I do not have the original computer files for adaptation to the format employed in the rest of the thesis. This format for presentation was approved by the Thesis Office, in verbal communication with Sylvia. My contribution to this study consisted of conducting a series of experiments examining the participation of the glycoprotein receptors GPIb and GPIIb-IIIa in the shear-associated aggregation of platelets activated with ADP. Monoclonal antibodies were used, with the antibody against GPIb specifically targeted against the von Willebrand factor binding site. The role of the receptors was examined at two shear rates. I feel that I have also contributed to some of the ideas presented in the discussion section. This section may also be considered to form an integral part of my studies of the role of vWF under varying agonist and shear rates.

Chapter 2

Surface-Secreted von Willebrand Factor Mediates Aggregation of ADP-Activated Platelets at Moderate Shear Stress: Facilitated by GPIb but Controlled by GPIIb-IIIa

Introduction to Chapter 3:

As we had been able to demonstrate a shear-dependent role for surface-expressed vWF in ADP-mediated aggregation under shear flow conditions in Chapter 2, we decided to examine specifically vWF-GPIb interactions in a shear-flow regime. Aggregation of platelets mediated by vWF and GPIb in the presence of a modulator, ristocetin, had previously been examined only under poorly defined dynamic conditions, in the stirred vials of an aggregometer. In the present study, we isolate the kinetics of ristocetin-mediated vWF-GPIb aggregation in shear flow using ristocetin, as a function of platelet-bound soluble vWF . Investigation of the role of surface-expressed vWF in shear-associated aggregation was extended to another physiologic agonist, thrombin. Thrombin concentration dependence for aggregation, vWF and P-selectin surface expression as well as GPIIb-IIIa activation were correlated. Results of this study were published in Thrombosis and Hemostasis 1998; 80: 428-36.

Chapter 3

Ristocetin and Thrombin-induced Platelet Aggregation at Physiological Shear Rates: Differential Roles for GPIb and GPIIb-IIIa Receptor

Abstract

We recently reported that washed platelets (WP) activated with ADP and expressing surface-bound vWF aggregated in flow through small tubes or in a cylindrical couette device at physiological shear rates of G =300 s^{-1} - 1000 s^{-1} in the absence of exogenous ligands, with GPIb-vWF partially, and activated GPIIb-IIIa totally required for the aggregation. We have now extended these studies to aggregation of platelets "activated" with ristocetin or thrombin. Washed platelet suspensions with added soluble vWF and ristocetin (0.3 - 0.75 mg/ml), or activated with thrombin (0.01 - 0.5 U/ml) but no added ligand, were sheared in a coaxial cylinder device at uniform shear rate, G =1000 s^{-1}. The collision capture efficiency (α_G) with which small aggregates form (= experimental/calculated initial rates of aggregation) was correlated with vWF platelet binding assessed by flow cytometry. The vWF-GPIb interaction was exclusively able to support ristocetin-mediated shear aggregation of metabolically active platelets, with very few vWF monomer equivalents bound per platelet (representing≤10 molecules of 10 million Da) required to yield high capture efficiencies (α_G= 0.38 ±.02; n=11), suggesting rapid and stable bond formations between vWF and GPIb. However, platelet surface-expressed vWF, generated by addition of thrombin to washed platelets, was found to mediate platelet aggregation with α_G = 0.08 ± .01 (n=6), surprisingly comparable to that previously reported for WP and ADP activation . Blocking the GPIIb-IIIa receptor decreased α_G by 95 ± 3% (n=3), while a monoclonal antibody to the vWF site on GPIb caused a 49 ± 7% (n=8) decrease in α_G. The partial role for GPIb thus appears to reflect a facilitative function for increasing contact time between flowing platelets, and allowing engagement of the GPIIb-IIIa receptor to yield stable attachment.

1. INTRODUCTION

Von Willebrand factor(vWF) is well adapted to a cross-bridging role between platelet and vessel wall, or platelet and platelet, due to its multimeric structure, with each repeating subunit containing binding sites to ECM proteins and to platelet glycoprotein receptors GPIb and GPIIb-IIIa. Metabolically active platelets adhere transiently to immobilized vWF, at shear rates up to G= 6000 s^{-1} via GPIb, with activated GPIIb-IIIa required for stable attachment (1), or through only the GPIb receptor if vWF is immobilized on a collagen-coated surface (2). Soluble vWF can mediate platelet aggregation in the absence of chemical agonists at shear rates of G≥6000 s^{-1} via both GPIb and GPIIb-IIIa (3,4). However, below these "pathological" shear rates, an external agonist is required. Ristocetin, or botrocetin mediate the binding of soluble vWF to GPIb on formalin-fixed platelets, while platelets activated with agonists such as ADP, thrombin or collagen, have been shown to bind soluble vWF via activated GPIIb-IIIa receptors, with vWF-mediated agglutination/aggregation in the stirred suspensions in the vials of the aggregometer, hereafter referred to as "stir" (5-9). Typical shear rates generated in aggregometer vials are estimated to be low (G≤ 30 s^{-1})(10), with variable flow patterns precluding theoretical calculations of shear aggregation kinetics and capture efficiencies. To date, the role of both soluble and secreted vWF, in the mediation of platelet aggregation at well-defined intermediate physiological shear rates in the presence of low levels of agonist, has not been investigated.

We have previously reported that ADP-activated washed platelets (WP), exhibiting increased surface-bound vWF can aggregate in the absence of exogenously-added ligands

at physiological shear rates, with a facilitative function postulated for GPIb, and an absolute requirement for GPIIb-IIIa (11). A role for surface-bound secreted fibrinogen(Fg) had been excluded in experiments by Goldsmith et al, where f $(ab')_2$ fragments of a polyclonal antibody against Fg failed to inhibit aggregation of ADP-activated WP in Poiseuille flow (12). A recent report has also demonstrated the dependence of aggregation on vWF-GPIb interactions in ADP and epinephrine activated platelets at pathologically high shear rates (G= 10,800 s^{-1}), in addition to a GPIIb-IIIa dependence, even in the presence of plasma Fg, with only a minimal role for the GPIb receptor found at the more physiological shear rate of G = 1200 s^{-1} (13). However, the role of secreted proteins was not evaluated. In the present study, we have extended our initial investigations using ADP and platelet surface-expressed protein mediated aggregation, to activation of WP with low concentrations of ristocetin, used to model vWF-GPIb interactions. Thrombin, an important physiological agonist expected to drive secretion to a greater extent than ADP, was also used to examine the relative roles of vWF-GPIb, and of vWF-GPIIb-IIIa interactions in supporting cross-bridging of platelets in laminar shear flow, under varying flow conditions, between G = 100 - 1000 s^{-1}.

2. MATERIALS AND METHODS

2.1. Reagents

Ristocetin A grade, ADP, α-thrombin and hirudin were purchased from Sigma Corp. USA; glutaraldehyde, electron microscopy grade, from Polysciences Inc.; paraformaldehyde from

Fisher Scientific Co., Fairlawn, NJ; human fibrinogen from Enzyme Research Laboratories Inc., South Bend, IN; H-Gly-Arg-Gly-Asp-Ser-Pro-OH peptide(GRGDSP) peptide, used to block the binding pocket of GPIIb-IIIa (14), from Calbiochem Corporation, La Jolla, CA; fluorescein-isothiocyanate (FITC; Isomer I) from Boehringer Diagnostics, La Jolla, CA). RO 44-9883, a non-peptidic analog, with greater specificity for GPIIb-IIIa than GRGDSP (15), was a gift from Dr. Beat Steiner from Hoffman-La Roche Ltd., Basel, Switzerland (called RO hereafter); ZK 36 374, a stable prostacyclin analog (16), was a gift from Dr. T. Krais, Schering Co., Berlin, Germany. Monoclonal antibodies (MoAbs) 6D1 , an IgG against the vWF binding site on GPIb (17) , and 10E5, an IgG against the GPIIb-IIIa receptor (18), were kindly donated by Dr. Barry S. Coller, State University of New York at Stony Brook; MoAb S.12, an IgG1, directed against platelet membrane CD-62P, P-selectin (19), was a gift from Dr. Rodger McEver, Oklahoma Medical Research Foundation, Oklahoma City, OK; MoAbs 2.2.9., an IgG1, recognizing an epitope, non-interfering with vWF binding to platelets, between residues 1366-2050 of vWF (8), and LJ-1b1, an $IgG_1{}^k$ recognizing the vWF binding site on GPIb(20) was generously provided by Dr. Z. Ruggeri, Scripps Institute, La Jolla, CA. Aurin tricarboxylic acid (ATA; 2900 Da. fraction), (21) which intereferes with the vWF-GPIb interaction (22) was provided by Dr. M. Weinstein (FDA, CBER, Rockville Pike, MD). Calibration beads, Quikcal: 7.1 μm, used for fluorescence intensity calibration of FITC-labelled proteins, were obtained from Flow Cytometry Standards Corp, Research Triangle Park, NC.

2.2 Preparation of von Willebrand factor

von Willebrand factor was purified from outdated cryoprecipitate obtained from the Canadian Red Cross according to established protocols (23). Briefly, a series of centrifugation steps was followed by application of the end supernatant to a Pharmacia Sepharose CL-4B column 100 x 0.9 cm. Fractions eluted at the void volume, comprising the ascending peak only, were pooled and the multimer distribution was verified with 1% agarose gels (24,25). 5% polyacrylamide gels of reduced vWF showed a predominant band at approximately 240,000 Da.

2.3 Preparation of washed platelets

Washed platelets(WP) were prepared by a one-step wash procedure from human citrated whole blood as previously described (12), involving acidification of platelet rich plasma (PRP) to pH 6.5 and by addition of 50 nM ZK, to inhibit platelet activation. The remaining pellet after centrifugation at 800xg for 15 minutes, was resuspended in half the original volume, in a modified Tyrode buffer (BAT:136 mM NaCl, 2.7mM KCl, 11.9 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 1.0 mM $MgCl_2$, 5.6 mM glucose, 0.35% BSA). Platelet suspensions were kept at 37° C under a mixture of 95% air and 5% CO_2 to maintain pH 7.4 (26).

2.4 Labelling of vWF and monoclonal antibodies

vWF was labelled with FITC according to the methods of Goto et al. (8). Briefly, vWF was diluted to 0.5 mg/ml with PBS (159 mM $NaHPO_4$, 37.8 mM NaH_2PO_4, 145 mM NaCl), pH 8.5 . FITC- Isomer I at 0.1 mg/ml, was added to vWF (0.1 mg. FITC per mg. protein),

incubated for 15 minutes, then dialysed against PBS. Protein concentration was calculated from absorbance measurements : OD_{280} - 0.35 OD_{495} / 0.7, to yield mg/ml. Fluorescein to protein ratios (F:P) were calculated by dividing the molar concentration of FITC by the molar concentration of FITC-vWF monomer equivalents (MW monomer ≈240,000 Da):

$$F:P = \frac{\text{moles fluorescein}}{\text{moles protein}} = \frac{[OD_{495} \div 5.56 \times 10^{-2}]}{[\text{FITC-vWF monomer equiv}]_{molar}}$$

Labelling of vWF resulted in molar F/P ratios between 1.1 and 3.5, thus producing good signal to noise ratios while maintaining high affinity ristocetin-mediated binding (8).

MoAbs S.12, 2.2.9, were labelled at concentrations of approximately 1 mg/ml., using FITC in DMF at ratios of 0.2 mg FITC per mg. protein, at a pH of 8.5 (27). Protein concentrations in mg/ml were calculated according to the formula OD_{280} - 0.35 OD_{495}/1.4, with F:P ratios calculated as above. Fibrinogen was labelled with FITC-Isomer I on Celite 10%, at pH 8.5, as previously described (28), and concentrations were calculated using the formula: OD_{280} - 0.286 OD_{495}/ 0.7, with F:P ratios calculated as above.

2.5 Binding experiments

Unless otherwise stated in Results, all binding studies of soluble vWF or antibodies to platelets were done under non-shear conditions.

2.5.1. Ristocetin-induced binding of soluble vWF to platelets; determination of numbers of vWF monomer equivalents bound at shear sampling times. Addition of ristocetin to

a suspension of washed platelets containing soluble vWF, results in rapid binding of vWF to platelets (29). Calculations of numbers of vWF monomer equivalents bound following 10-20 seconds of shear, were based on handling times required for addition of ristocetin/vWF to platelet suspensions, transferring of the suspension to the microcouette, zero-time and 10 s subsampling, corresponding typically to an additional 30 seconds (total of ~ 50 s for 20 s of shear). Since we expected that the low concentrations of ristocetin and vWF used for shear would result in low fluorescence values for bound vWF, we estimated numbers of vWF bound in two steps: determination of 1) maximal equilibrium binding with the actual concentrations of vWF and ristocetin used for shearing, and 2) percentage of maximal binding at actual subsampling times following shear, using the time-course binding of a higher concentration of FITC-vWF (15 μg/ml) yielding measurable fluorescence values for FITC-vWF bound.

2.5.2. Equilibrium binding. Washed platelets (WP) at a concentration of $10^4/\mu$l, in BAT buffer, with 1 mM Ca^{2+} added immediately prior to incubation, were incubated for 30 minutes with decreasing concentrations of ristocetin as used for shear experiments: 0.75, 0.5 or 0.3 mg/ml, and 5 μg/ml vWF, to allow equilibrium binding. The reaction was arrested by diluting platelets with 10 volumes of BAT buffer and the average fluorescence per platelet particle was read immediately on a flow cytometer, FACScan (Becton Dickinson, Mississauga, Ont), as previously described (30). Platelet samples were analysed for 3,000 cells. Samples were excited using an air-cooled argon ion laser emitting light at 488 nm. Lysis II software was used to calculate forward scatter (FSC), side scatter (SSC) and FITC-

fluorescence (FL-1) data. Platelets were identified on the basis of characteristic FSC/SSC profiles and gates were set to exclude aggregates from platelet populations used to generate fluorescence histograms. Numbers of FITC-vWF monomer equivalents bound (with monomer equivalent weights ≈240,000 Da), were determined from average fluorescence values, using MESF Quikcal calibration beads, from the equation: 220(fluorescence) - 1099/ F:P, as previously reported for FITC-Fg (28) . The specificity of FITC-vWF binding to platelets was ascertained using MoAb 6D1, which completely inhibited all ristocetin-mediated binding, as did addition of an excess of unlabelled vWF. In the case of binding experiments at ristocetin concentrations of 0.3 mg/ml, the fluorescence values were at the limit of FACScan sensitivity, i.e. <5 Fl units. Hence, application of the above equation would result in negative values, prohibiting calculation of the numbers of monomer equivalents bound. In these cases, numbers of vWF monomer equivalents bound are indicated as < 300, which corresponds to FL = 6.

2.5.3. Time-course binding. To WP (10^4 /μl) were added Ca^{2+} (1 mM), ristocetin (0.5 mg/ml) and FITC-vWF(15μg/ml) in a total volume of 20 μl, and incubated at room temperature for 10, 30, 60 seconds or 2,3,5,10,20,40 minutes. The reaction was arrested by a 10-fold dilution with BAT buffer, and results were read immediately on the FACScan.

2.5.4. Binding to thrombin-activated WP. In order to assess effects of activating WP with different concentrations of thrombin, FITC-2.2.9, FITC-S.12 and FITC-Fg, were used to report on the amount of surface-expressed vWF (SE-vWF), α-granule secretion and platelet

GPIIb-IIIa activation state, respectively. 5μl of WP, at 4 x 10^4 /μl in BAT buffer, were added to FACS tubes with thrombin at concentrations between 0.008 U/ml to 0.5 U/ml and incubated for 20 minutes in a total volume of 20μl, containing buffer, 1mM Ca^{2+} and either 530 nM FITC-2.2.9, or 273 nM FITC-S.12. For binding with Fg, thrombin-activated WP were first incubated for 10 minutes, neutralized with hirudin at a ratio of 4:1, and 0.5μM FITC-Fg was then added and incubated for a further 20 minutes. Following incubation, suspensions were diluted with 10 vols. of Ca^{2+} -free BAT buffer, and analysed immediately on the FACSCAN. The binding of fluorescently-labelled ligand to WP for each thrombin concentration was determined for FITC-2.2.9, FITC S.12, and FITC-Fg. Subpopulations showing bound FITC-2.2.9 or FITC-S.12 expressed maximal numbers of surface-bound vWF or P-selectin respectively, or in the case of FITC-Fg, subpopulations expressed increasing numbers of FITC-Fg bound as a function of increasing thrombin concentration. The fraction of platelets expressing fluorescence values greater than control levels for unactivated platelets, will henceforth be referred to as the percentage activated platelet population (%P*). In a separate series of experiments, WP prepared from the same donor were used to compare the response to increasing concentrations of thrombin by evaluating in parallel, the surface expression of vWF, as reported by MoAb 2.2.9, in the absence of shear, as well as shear-associated aggregation.

To examine inhibition of thrombin-induced binding of vWF to the platelet surface, as reported by FITC-2.2.9, incubation conditions were as above, except that inhibitors were added immediately prior to addition of 0.05 U/ml thrombin: 67 nM 6D1, 67 nM 10E5, 500 nM RO or 29 μM polymeric ATA, 2900 Da fraction, concentrations which we had

determined to effectively block ligand-GPIIb-IIIa or ristocetin-mediated vWF-GPIb interactionsr . Results were read on the FACScan as above.

2.6. Aggregation in flow device

Aggregation of washed platelets in laminar shear flow was tested in a microcouette (coaxial cylinder device), with dimensions and properties similar to those previously described (31), but with outer and inner (R1) cylinder of radius 7.3 and 7.0 mm respectively. The inner cylinder was rotated at variable angular velocity, ω, corresponding to shear rates G, given by $G = R_1\omega/h$, from 1 -1000 s^{-1} ($h<<R$), by means of a high precision step motor. Subsamples were collected at fixed time intervals of shear (0, 10, 20, 60 sec) after arresting the motor. They were drawn from a port on the outer cylinder situated just above the base of the inner cylinder. The first 6 μl, constituting dead volume, were discarded, then twenty microliter aliquots were drawn and immediately fixed in 10 vol of 0.8% glutaraldehyde. These samples were further diluted 7-fold with isotonic saline, to permit analysis of particle number with the FACScan, by counting the number of particles acquired in twenty seconds, assuming a constant flow rate. Platelet aggregation(PA) was calculated by determining the decrease in particle number with time: $\% PA = (1 - N_t/N_0) \times 100\%$, where N_t = platelet particle number at time t, and N_0 is the initial platelet number. As previously described (31), platelet collision capture efficiencies, α_G , defined as the ratio of the rate of two-body collisions resulting in aggregate formation to the total rate of two-body collisions, were computed using equations given by Smoluchowski (32), assuming dilute suspensions of rigid spheres, from the ratio of experimental/calculated initial rate of aggregation.

2.6.1. Aggregation of WP induced by ristocetin or thrombin. Washed platelet suspensions of 5 x $10^4/\mu$l were pre-mixed in Eppendorf tubes with BAT buffer containing 1mM Ca^{2+}, 5 μg/ml vWF, and 0.3, 0.5 or 0.75 mg/ml ristocetin, then immediately transferred to the microcouette for shear experiments. For activation with thrombin, platelets were incubated with varying concentrations of thrombin for 10 minutes, then transferred to the microcouette, since the longer incubation was previously demonstrated to result in higher binding of secreted vWF to platelets (33), and confirmed by us to result in an increased extent and rate of aggregation. In inhibition studies, platelet suspensions were pre-incubated for 0.5 - 5 minutes in the presence of either 67 nM 6D1, 67 nM 10E5, 100-600 μg/ml polyclonal anti-vWF or isotypic control, 500 nM RO 44-9884 or 29 μM polymeric ATA, 2900 Da fraction, prior to addition of agonist.

3. RESULTS

3.1. Aggregation mediated by ristocetin

Washed platelets, to which 0.3, 0.5 or 0.75 mg/ml ristocetin had been added, were sheared in the presence of 5μg/ml vWF in the microcouette at shear rates G = 100, 300, and 1000 s^{-1}. At any given shear rate, the maximum extent of aggregation following 60 seconds of shearing was the same at each of the three ristocetin concentrations tested, but varied with increasing shear rate (Fig.3-1), from 50 ± 4%(n=10) at G = 100 s^{-1}, to 75 ± 1%(n=9) at G

$= 300\ s^{-1}$ *, to 86 ± 1%(n=11) at G = 1000 s^{-1} * (* = p<.001), where data have been pooled for experiments at the three ristocetin concentrations. Capture efficiencies, α_G, determined from initial rates of shear-associated aggregation of WP and soluble vWF, were also independent of ristocetin concentration, but α_G was significantly lower (by 16 %) at G = 1000 s^{-1} compared to G = 100 s^{-1}, the values being α_G = 0.45 ± .01(n=9), 0.43 ± .01(n=8), 0.38 ± .02(n=11)**(**=p <0.05) at G = 100, 300 and 1000 s^{-1} respectively. These capture efficiencies are surprisingly high for all shear rates tested, even at G = 1000 s^{-1}, where for fibrinogen-driven shear aggregation, α_G values had previously been shown to be about six-fold lower (31).

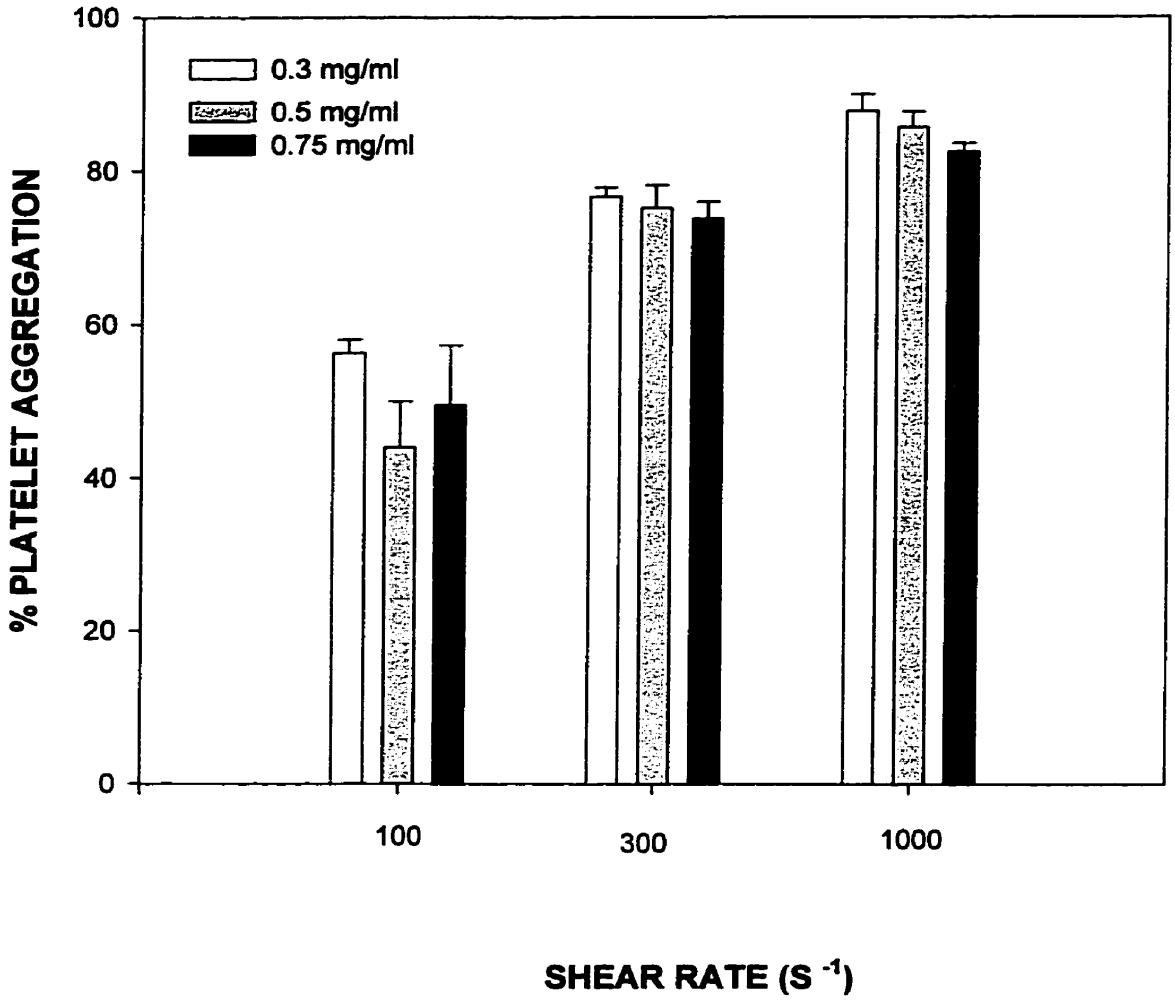

FIGURE 3-1. Maximum platelet aggregation of washed platelets (WP) with varying ristocetin concentrations and shear rates. WP were pre-mixed with BAT buffer, vWF and 0.3, 0.5 or 0.75 mg/ml ristocetin, then transferred to a microcouette for shearing at G = 100, 300 or 1000 s^{-1}. Bars show mean ±SEM of at least 3 separate experiments, done in duplicate.

From the measured time-course of diffusion-driven FITC-vWF binding, we expect < 10 % of maximal vWF to bind in the ~ 30 s delay following ristocetin addition before the onset of shear, and < 30% of maximal binding in the first 10- 20 s of shear. From Table 3-1, correlating capture efficiencies and numbers of vWF monomer equivalents bound after 20 s of shear, we can see that there is at least a 3-fold increase in the number of vWF monomer equivalents bound at 0.3 vs 0.5 mg/ml ristocetin (Fl values: < 300 vs 990 ± 105 (n=3)) and a further doubling at 0.75 mg/ml ristocetin (2035,(n=2)). Surprisingly, vWF is able to cross-bridge platelets with the same high efficiencies, even at the lowest numbers of vWF monomer equivalents bound : < 300, corresponding to ≤ 10 molecules of 10 million Da.

Table 3-1. Relation between number of vWF monomer equivalents bound per platelet and the capture efficiencies (α_G) for ristocetin-mediated shear aggregation[a].

RISTOCETIN CONCENTRATION (mg/ml)	VWF BOUND[b] PER PLATELET	CAPTURE EFFICIENCY (α_G)
0.3	<300	0.449 ± 0.003
0.5	990 ± 105	0.425 ±0.016
0.75	2035±409	0.422 ± 0.022

[a] Washed platelets were sheared at G = 1000 s^{-1} in the microcouette with ristocetin and soluble vWF. α_G was calculated as described in the Methods.Results are presented as mean ± SEM.

[b] Estimated as vWF monomer equivalents bound, after 20 s shear.

3.1.1. Inhibition of platelet receptors. Washed platelet suspensions to be used for aggregation were incubated with monoclonal antibodies 6D1 and 10E5 to block the vWF-binding site on GPIb and the RGD recognition site on GPIIb-IIIa, respectively; with polymeric ATA, 2900 Da fraction, to block vWF-GPIb interactions (22) and having possible anti-GPIIb-IIIa effects (34); or with RO 44-9883 (RO), to specifically block the GPIIb-IIIa receptor (15). Blockers of the GPIIb-IIIa receptor had no effect on WP aggregation mediated by soluble vWF and ristocetin (fig.3-2); % Inhibition (%I) = 0 and 4% for MoAb 10E5 and RO respectively, suggesting that within 60 seconds of shear , the aggregation of WP was entirely independent of the GPIIb-IIIa receptor. Pre-incubation of platelets with ristocetin and vWF for up to 10 minutes did not change the inhibition characteristics (results not shown). In contrast, the 6D1 MoAb completely blocked platelet aggregation, suggesting that the ristocetin-mediated aggregation, characterized by a high capture efficiency, required vWF interaction with the GPIb receptor alone.

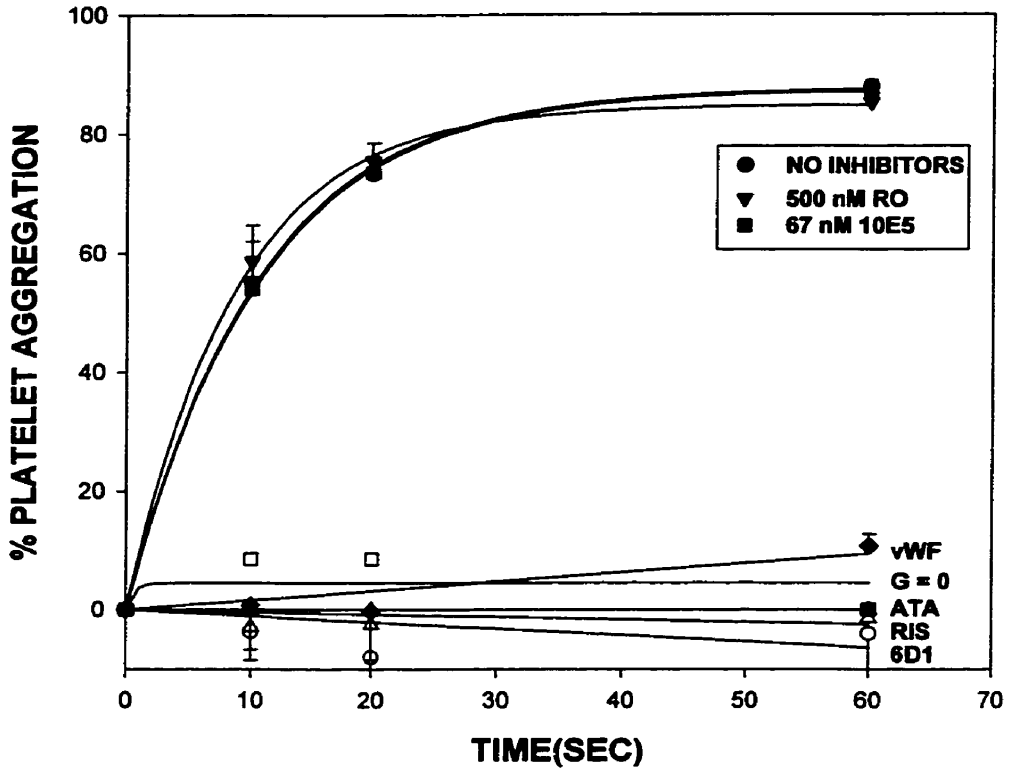

FIGURE 3-2. Time-course of vWF-ristocetin-mediated shear aggregation of WP at G = 1000 s^{-1}, and effect of inhibitors. Platelet suspensions were incubated for 3 minutes with buffer (●), or with inhibitors RO 44-9883 (RO)(▼) or MoAb 10E5 (■), against glycoprotein GPIIb-IIIa; MoAb 6D1 (○) against glycoprotein GPIb; or polymeric ATA 2900 Da. fraction (▲), with effects expected against vWF-Ib interactions, and possibly against GPIIb-IIIa interactions. vWF and ristocetin were then added and the whole suspension was transferred immediately to the microcouette for shearing. A negative control was run with ristocetin and no vWF (◆), vWF and no ristocetin (△), and ristocetin and vWF but no shear (□);. Samples were drawn at t= 0, 10, 20 and 60 s. Data represent means ±SEM of 3 separate experiments, done in duplicate.

3.2. Platelet activation and aggregation mediated by thrombin:

Studies by Frojmovic et al (11) have shown that ADP activated WP have increased surface-bound vWF and can aggregate in a uniform shear field, with an absolute requirement for GPIIb-IIIa and a partial requirement for GPIb. Here, we have extended the work to washed platelets activated with thrombin. WP pre-incubated with thrombin concentrations between 0.008 to 0.5 U/ml were sheared at G = 1000 s^{-1} in the microcouette, in order to investigate the dependence of aggregation on thrombin concentration. At thrombin concentrations ≤ 0.01 U/ml, the aggregation observed (<10%) was not significantly different from control runs without thrombin (Fig. 3-3). At concentrations of 0.05 (or 0.02 U/ml; not shown, depending on the donor), concentrations at which thrombin is expected to activate platelets mostly through its high affinity binding sites on GPIb (35), there was a threshold response, with %PA = 61 ± 7% (n=4), representing 83% of the maximum % PA obtained at 0.5 U/ml.

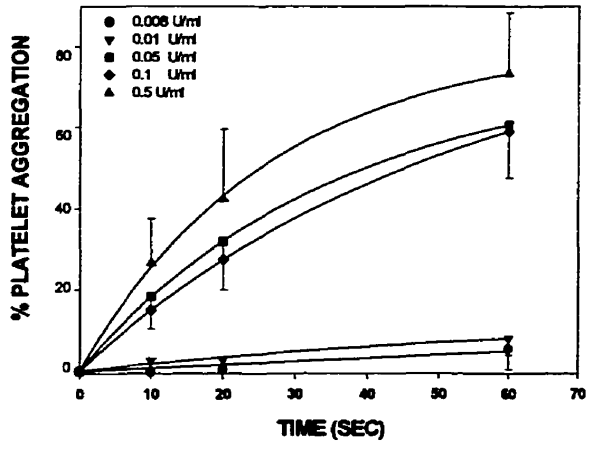

FIGURE 3-3. Kinetics and extent of platelet aggregation as a function of thrombin concentration. Washed platelet suspensions (4x 10^4 /μl), were activated with thrombin at concentrations between 0.008 U/ml to 0.5 U/ml, and incubated for 10 minutes in Eppendorf tubes, prior to transfer to the microcouette for shearing at G = 1000 s^{-1}. Results shown are mean ±SEM for four experiments, done in duplicate.

3.2.1. Effect of thrombin on platelet secretion, surface-bound vWF and activation state of GPIIb-IIIa. The effect of shear on the expression of epitopes for MoAbs 2.2.9 and LJ-1b1, reporting on SE-vWF and GP-Ib, was evaluated for WP activated with 0.05 U/ml thrombin, in suspensions too dilute for aggregation to occur over the time period tested (< 6000/ μl; $PA_{max} \leq 5$ %). Exposure of platelets to shear rates between G = 300 - 2000 s^{-1} for 60s, resulted only in -7 % and 8 % (n = 2) differences in SE-vWF and GPIb receptors, respectively, relative to unsheared platelets.Thus further binding studies were done under non-flow conditions.

Washed platelets were incubated under equilibrium binding conditions with varying thrombin concentrations and either FITC-labelled MoAbs 2.2.9 to report on surface-bound vWF, MoAb S.12 to report on α-granule P-selectin, reflecting fusion of α-granule membrane with the plasma membrane and secretion of granule contents, or FITC-fibrinogen (Fg) to report on the activation state of the GPIIb-IIIa receptor, to determine if we could find a correlation between the minimum thrombin concentration required for platelet aggregation, and a significant, sudden threshold increase in binding of either antibodies or of Fg. Indeed, as illustrated in Fig.3-4 for a representative donor, there was a strong correlation between the threshold response of FITC-2.2.9 binding and that of the capture efficiency, both occurring at the minimal thrombin concentration required for aggregation (0.02 to 0.05 U/ml thrombin, depending on the donor).

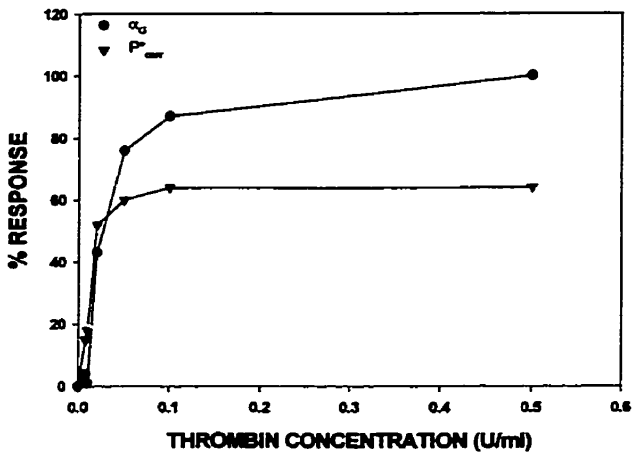

FIGURE 3-4. Percent dose- response of α_G, and percent platelet population showing maximal response for surface-bound vWF (% P*), for thrombin-activated WP. WP activated with increasing concentrations of thrombin were used for both shear aggregation studies ($4x10^4/\mu l$) at G = 1000 s^{-1}, as described for fig. 3-3 above, and for binding studies ($1 \times 10^4 / \mu l$) with vWF reporting antibody FITC-2.2.9. P^*_{corr} represents P* corrected for background fluorescence of FITC-2.2.9 on untreated platelets. Capture efficiencies varied between 0.08 and 0.162 depending on the donor. Figure is representative of results obtained for 3 donors.

Thus, the % of activated platelets exhibiting maximal surface-expressed vWF (%P*), increased at the threshold point from 15 ±3%(n=3)(no thrombin) to 73 ±4% (n=3) with 0.02 - 0.05 U/ml thrombin. Furthermore, the % P* was consistently larger than the % of platelets showing α-granule secretion (P_s expression)(19 ±6%(n=3)), or GPIIb-IIIa activated receptors (29±13%(n=3)), the latter determined by FITC-Fg binding at 0.02 - 0.05 U/ml thrombin (Fig. 3-5). In addition, EC_{50} thrombin concentrations were two and three-fold lower for FITC 2.2.9 than for FITC S.12 and for FITC-Fg, respectively (Table 3-2).

Table 3-2. EC_{50} for thrombin causing surface-bound vWF secretion and GPIIb-IIIa activation as reported by FITC 2.2.9, S.12 and Fg binding to platelets.

DONOR	EC_{50} [a] FOR THROMBIN-INDUCED BINDING OF:		
	FITC- 2.2.9(U/ml)	FITC- S.12(U/ml)	FITC- Fg(U/ml)
1	0.012	0.038	0.058
2	0.031	0.041	0.037
3	0.017	0.03	0.043
X ± SEM(3)	0.020±0.005	0.036±0.003(*)[b]	0.046±.005 (*)[b]

[a] Washed platelets were incubated with monoclonal antibodies FITC- 2.2.9 and FITC- S.12, or FITC-Fg to report on surface-bound vWF, P-selectin (indicating α- granule secretion) and GPIIb-IIIa activation respectively. The effective concentration (EC_{50}) for thrombin was determined for these parameters, incubated with WP as described for Fig.6.

[b] Paired t- test, compared with FITC-2.2.9; * corresponds to $P < 0.05$

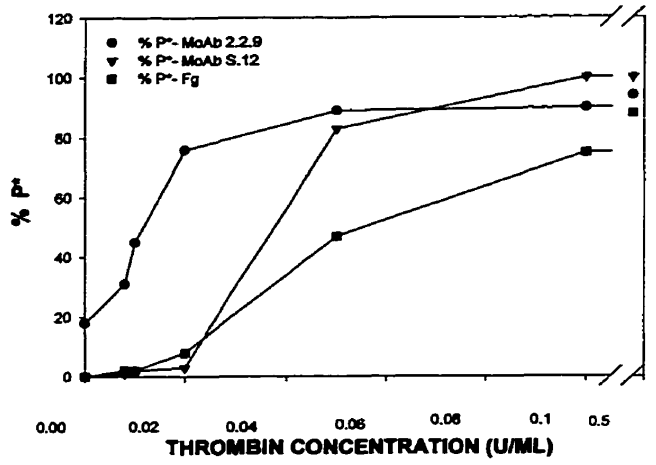

FIGURE 3-5. Thrombin-induced changes in parameters of platelet activation, represented as % population showing maximal response (%P*). FITC-2.2.9, FITC S.12 and FITC-Fg were used to report on the amount of secreted, surface-bound vWF, platelet P-selectin (indicating α-granule secretion) and GPIIb-IIIa activation state, respectively. WP ($1 \times 10^4 / \mu l$) were activated with increasing concentrations of thrombin and incubated with labelled MoAb's 2.2.9 and S.12 approx. 20 minutes, or with Fg following 10 minutes of thrombin activation and 5 minutes neutralization with hirudin. Data shown are mean ±SEM of 3 separate experiments, done in duplicate.

3.2.2. Inhibition of thrombin-induced aggregation: Platelets were pre-incubated with 0.05 U/ml thrombin and with inhibitors for ten minutes before being transferred to the microcouette and sheared at $G = 1000\ s^{-1}$. MoAb 10E5 and RO, which block the adhesive domains on GPIIb-IIIa receptors for ligands like vWF and Fg, essentially reduced the aggregation capture efficiency from $\alpha_G = 0.08 \pm .01 (n = 6)$ to virtually zero, reflecting a complete inhibition of initial rates of aggregation (%I = 97 ± 3% (n = 3), and 98 % (n = 2)

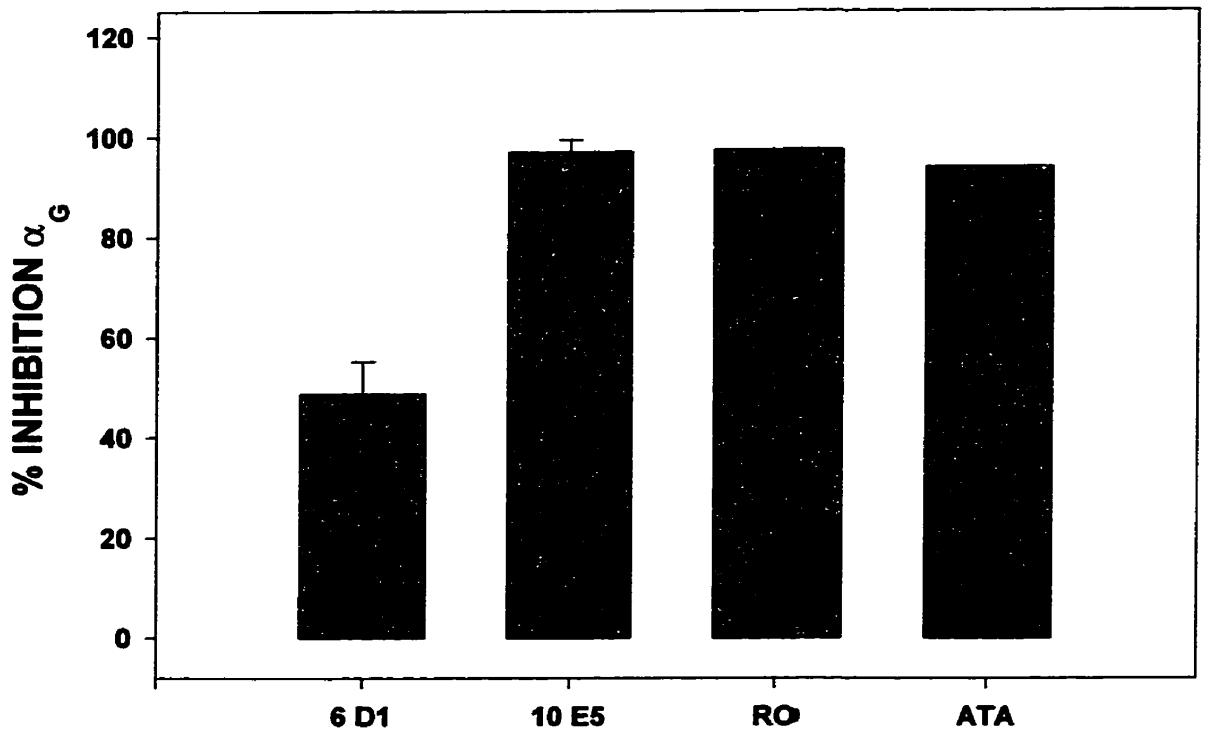

FIGURE 3-6. Inhibition of capture efficiency for thrombin-induced shear aggregation at G= 1000 s^{-1}. Washed platelets were pre-incubated with inhibitors against GPIb and GPIIb-IIIa (as used in Fig. 2), and 0.05 U/ml thrombin for 10 minutes in Eppendorf tubes, prior to tranfer to the microcouette for shearing. Capture efficiencies were calculated as described in "Methods", with control platelet runs lacking inhibitor set to α_G=100% (α_G = 0.08 ± .01). Data shown are means ±SEM for 2 to 3 separate experiments, done in duplicate.

respectively; Fig.3-6). ATA was equally effective, inhibiting α_G by (94%(n=2)). The MoAb 6D1 was also able to reduce α_G by 49±7% (n=8), pointing to the importance of the GPIb receptor for initial capture of platelets at this intermediate shear rate, similar to that previously reported for ADP-induced aggregation of WP (11). Control shear experiments testing for a platelet concentration dependent inhibition by MoAb 6D1 of sheared WP activated with 0.05 U/ml thrombin, showed < 10% difference in the extent of inhibition by MoAb 6D1 of initial rates of aggregation, between platelets at an initial concentration of 5 x $10^4/\mu$l versus 1x $10^5/\mu$l. No loss of 6D1-GPIb complexes as a result of internalization of GPIb receptors was expected, since at the low thrombin concentrations used, the GPIb receptor was not downregulated, as determined via MoAb LJ-1B1 (results not shown).

A polyclonal anti-vWF antibody showed a dose-dependent inhibition of platelet aggregation at G = 1000 s^{-1}, with a maximum inhibition of α_G at 600 μg/ml (83±6 %), and no inhibition using an isotypic control or buffer (Fig.3-7).

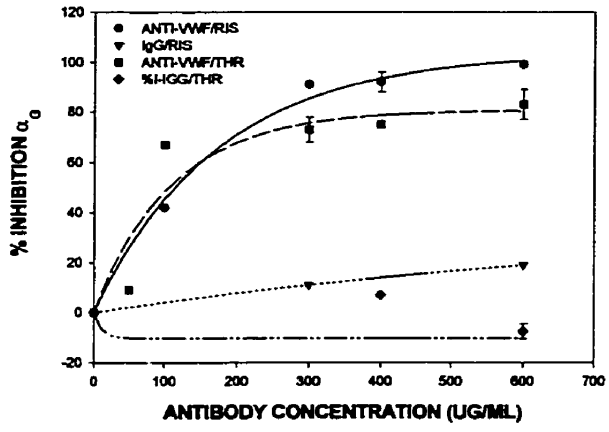

FIGURE 3-7. Dose-dependent inhibition of capture efficiencies for ristocetin (RIS) and thrombin (THR)-induced shear aggregation of WP at G = 1000 s^{-1} by polyclonal anti-vWF antibody. Antibody (or control IgG) was added to platelet suspensions immediately before addition of agonist, then either transferred immediately to the microcouette for ristocetin-mediated shear experiments, or incubated for 10 minutes with thrombin prior to shearing.Capture efficiencies (α_G) in the absence of inhibitor were 0.45 ± .02 (n=3) and 0.09 ± .02 (n=4) for ristocetin and thrombin experiments, respectively.

This was compared with the inhibition of α_G for vWF-ristocetin-mediated aggregation by the antibody where, in a solely vWF-mediated system, it is expected to block aggregation. At 600 μg/ml, the % max inhibition was 99%, but the isotypic control also inhibited aggregation by approximately 19%. Thus, the net inhibition of the ristocetin-vWF system was only about 80%, similar to that for thrombin-induced aggregation of WP under shear, and we could not determine whether this was due to non-specific interactions of ristocetin with the high levels of antibody, or to other causes. Nevertheless, it is clear that at the shear rate tested, and for washed platelets activated at low thrombin concentrations, it is vWF which is the predominant cross-bridging protein, rather than some other surface-bound secreted protein, with both GPIb and the GPIIb-IIIa receptors playing an important role.

3.2.3. Effects of inhibitors of GPIb and activated GPIIb-IIIa on thrombin-induced surface-bound vWF. We incubated washed platelets activated with 0.05 U/ml thrombin with various inhibitors under non flow, equilibrium conditions. Monoclonal antibody (MoAb) 6D1, interfering with the vWF-GPIb interaction and used at concentrations which we found inhibited ristocetin-mediated binding, had little effect on 0.05 U/ml thrombin-induced binding of vWF reporting MoAb FITC-2.2.9 (%I = 11 ± 7 (n=6); results not shown), while inhibiting the GPIIb-IIIa receptor with the MoAb 10E5 or with RO resulted only in a 9 ± 4 %(n=5) decrease and a 5 ± 3% (n=3) increase in FITC-2.2.9 binding respectively. Polymeric ATA, with effects on GPIb and GPIIb-IIIa, essentially blocked all FITC-2.2.9 binding, as indeed it had blocked platelet aggregation (Fig. 3-6).

4. DISCUSSION

The shear regime to which cells are subjected may dictate cell interactions directly through a physical modification of receptors or ligands (36). Alternatively, shear flow may affect the times spent by colliding cells in close proximity, and thereby affect the relative importance of specific proteins mediating capture of cells, depending on their ligand-receptor affinities and relative rates of bond formation (1,10,37) In the absence of exogenously added chemical agonists like ADP or thrombin, soluble vWF plays a predominant role in cross-bridging platelets at pathologically high shear rates ($G \geq 6000\ s^{-1}$)(3,4,38). In this study, we have attempted to investigate vWF-platelet interactions at more physiological shear rates, and to specifically isolate the kinetics of vWF-GPIb mediated shear aggregation using ristocetin. We then investigated the aggregation kinetics of a more physiological system with thrombin activation, in the absence of exogenous proteins, but with major secretion and surface expression of ligands like vWF.

4.1. Role of the GPIb receptor in soluble vWF-ristocetin-induced aggregation:

We have shown that in the presence of a modulator, ristocetin, and soluble vWF, very few vWF monomer equivalents bound to platelets are required for cross-bridging at physiologically intermediate shear rates between $G = 100 - 1000\ s^{-1}$. The shear aggregation observed is independent of GPIIb-IIIa, as evidenced by the inability of RO and MoAb 10E5 to inhibit the reaction, but is completely inhibited by an antibody blocking interactions with the glycoprotein receptor GPIb-IX-V, strongly suggesting that vWF-GPIb interactions alone are sufficient to maintain aggregation of metabolically active platelets. These findings are

perhaps surprising since ristocetin-mediated binding of vWF to the GPIb receptor can cause signalling and activation of the GPIIb-IIIa receptor (39), and a requirement for GPIIb-IIIa for maintenance of stable aggregates was shown in stirred suspensions of Glanzmann's Thrombasthenia patient platelets "activated" with ristocetin (40). However, under shear flow, there may be exposure of both additional vWF subunits and specific epitopes for GPIb due to shear-induced uncoiling of vWF(36), so that an initially weak ristocetin-mediated vWF-GPIb interaction, may be strengthened by multiple interactions with vWF repeating subunits. Thus, within the time frame of exposure to shear ($\leq$ 1 min), the ristocetin-vWF mediated aggregation can be expected to provide a valid model of an exclusively vWF-GPIb system (similar to GPIb-only adhesion of platelets under shear to vWF-coated onto collagen (2)), with capture efficiencies (α_G) calculated accordingly reflecting only vWF-GPIb interactions.

The α_G 's obtained at G = 1000 s^{-1} ($\approx$0.4) for ristocetin-soluble vWF mediated platelet aggregation, indicate that vWF-GPIb interactions, as modulated by ristocetin, allow capture of colliding platelets with high efficiency. This efficiency is approximately six times higher than for ADP-fibrinogen mediated aggregation at similar shear rates (31). It thus appears that vWF-GPIb bonds must be rapidly formed in order to mediate capture of platelets in flow, since mean contact times available between two platelets at G = 1000 s^{-1} are estimated to be short, $t_c \approx 5\pi/6G \approx$ 2.6 msecs, assuming spherical geometry (41). The ability of vWF and GPIb alone, to mediate surprisingly efficient aggregation without participation of GPIIb-IIIa, but with appropriate vWF conformations, could provide a paradigm for vWD Type IIB patient thrombocytopenia, where increased affinity of vWF may allow spontaneous binding to GPIb, efficient cross-bridging of platelets, and hence stable aggregation in shear.

4.2. Role of the GPIb and GPIIb-IIIa receptors in thrombin activated WP :

Surface-expressed vWF has been shown to be involved in the mediation of aggregation of washed platelets activated with 5μM ADP (11), in the absence of exogenous ligands, where there was a complete requirement for the activated GPIIb-IIIa receptor, and a partial requirement for the GPIb receptor. Here we extended these studies to the investigation of thrombin activated washed platelets, where, under non-flow conditions, soluble vWF reportedly binds exclusively to the GPIIb-IIIa receptor (8,35).

We have shown that for washed platelets activated with thrombin and sheared in the absence of exogenous soluble proteins: 1) there is a thrombin concentration threshold for the onset of aggregation at physiologically intermediate shear rates; 2) this threshold is correlated with the % of platelets having maximal surface-expressed vWF (%P*-vWF), but relatively low % of platelets with α-granule membrane fusion (probed for P-selectin expression) or activation of GPIIb-IIIa; 3) under shear flow, initial rates of aggregation are at least partly dependent on GPIb and totally on GPIIb-IIIa; and 4) vWF is the predominant, if not exclusive, adhesive protein mediating aggregation via surface expression on the activated platelet.

The existence of a threshold concentration for the onset of thrombin-induced aggregation in stirred suspensions (aggregometer) of PRP or WP was previously reported (42) and verified in our lab, but hitherto had not been tested at the physiologically intermediate shear rates examined here in couette flow. We found that the threshold occurred at thrombin concentrations similar to those reported in the aggregometer, unlike the case for ADP, where

Bell et al. have shown that five-fold lower concentrations of ADP were sufficient for driving aggregation of PRP under Poiseuille flow at 22^0 C than in the aggregometer at 37^0 C (43,44). This difference was not attributable to temperature, since the kinetics of aggregation in the aggregometer were shown to be similar at 37^0 C and 22^0 C (45).

The necessary role for vWF in thrombin-mediated aggregation is supported by the greater than 80% inhibition of aggregation obtained with a polyclonal anti-vWF antibody. It was previously shown that aggregates of washed platelets activated with thrombin and stirred in aggregometer vials showed minimal released fibrinogen at sites of close platelet contact in the first 20 s following thrombin-induced aggregation (46,47), suggesting that secreted fibrinogen was not important for initiating cross-bridging of platelets, although Fg appeared by 40 s and GPIIb-IIIa complexes were continually located between adjacent surface membranes throughout the aggregate. Thus, the possible role of protein ligands other than vWF, like Fg and TSP, is currently being investigated. The lack of inhibition of expression of vWF on the platelet surface by specific anti GPIIb-IIIa antagonists, indicate that they did not affect thrombin-induced aggregation via any direct inhibition of surface-expressed vWF on individual platelets, but rather by occupying free sites on GPIb or GPIIb-IIIa.

The similarities in capture efficiencies (α_G) and their inhibition by the anti-GPIb MoAb 6D1, for shear aggregation studies with ADP and thrombin-activated WP, suggest that similar mechanisms are operating in both systems at intermediate shear rates: vWF likely interacts with GPIb to facilitate capture of platelets with subsequent cross-bridging via vWF-GPIIb-IIIa. The rapidly formed but easily ruptured vWF-GPIb bonds, have been shown to be an efficient means of capturing platelets in flow, either in our ristocetin-mediated

aggregation of platelets at moderate shear rates ($\alpha_G \approx 0.4$), or for surface-immobilized vWF at shear rates tested in excess of 6000 s^{-1} (1). We propose therefore, that at intermediate to higher G, vWF pre-bound to GPIb or GPIIb-IIIa can initially cross-bridge via the GPIb receptor, thereby increasing contact time between platelets, and allowing more stable, but slower occurring bonds to form between vWF and GPIIb-IIIa. Although soluble vWF is considered to be unable to bind to GPIb at shear rates below $G \approx 6000\ s^{-1}$ without intervention by an agonist (4,8), the surface-bound secreted vWF, essentially in an "immobilized" conformation, is expected to present epitopes for recognition by GPIb on another platelet. Direct physical forces due to shear may play a role as well. In studies by Dong et al. (48), CHO cells expressing recombinant GPIb-IX, were shown to agglutinate in the presence of soluble vWF without ristocetin, when strongly agitated, due to physical forces alone.

The more "sensitive" exposure of surface-bound vWF on washed platelets activated with threshold thrombin concentrations, prior to full secretion of other proteins, as suggested by delayed P-selectin exposure, and prior to GPIIb-IIIa activation (Fig.3-5), could allow vWF to access optimal numbers of unoccupied GPIb and GPIIb-IIIa receptors for mediating platelet aggregation. The surface-expressed vWF may be secreted pre-bound to GPIb, since vWF has been shown to co-localize with GPIb receptors in α- granule membranes and in the surface-connected canalicular system (49), or may be derived from a non α-granule vWF pool, e.g. of cytoplasmic or surface-connected canalicular system origin (50,51).The differential secretion of vWF may even be a result of an asymmetric budding from α-granules for fusion with plasma membrane, of vWF-containing regions, where vWF is eccentrically localized as tubular structures (49). This would yield low P-selectin to vWF

ratios expressed on the platelet surface, as we have actually observed.

Thus, we have shown that vWF plays an important role in cross-bridging of platelets in ristocetin-mediated shear aggregation, through the GPIb receptor exclusively, with low numbers of soluble vWF monomer equivalents bound (representing≤ 10 molecules vWF of 10 million Da). Additionally, surface-expressed vWF expressed on platelets activated by low concentrations of thrombin, can mediate cross-bridging at intermediate shear rates in the absence of exogenous proteins, dependent on both GPIb and GPIIb-IIIa receptors. This is in contrast to the minimal role described for the GPIb receptor in studies at G= 1200 s^{-1}, for ADP and epinephrine activated platelets (13), where the contribution from secreted proteins is likely less, though this was not directly evaluated. Thus, thrombin-induced surface-expressed vWF may provide an additional highly efficient mechanism for platelet aggregation in the early stages of hemostasis where only very low concentrations of thrombin are present to activate platelets.

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6. REFERENCES

1. Savage B, Salvidar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. Cell 1996; 84: 289-97.

2. Lankhof H, Wu YP, Vink T, Schiphorst ME, Zerwes HG, deGroot P, Sixma JJ. Role of the glycoprotein Ib-binding A1 repeat and the RGD sequence in platelet adhesion to human recombinant von Willebrand factor. Blood 1995; 86: 1035-42.

3. 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 GPIb in the initiation of shear-induced platelet aggregation. Thromb Haemost1993; 69: 496-502.

4. Moake JL, Turner NA, Stathopoulos NA, Nolasco LH, Hellums JD. Shear-induced platelet aggregation can be mediated by vWF released from platelets, as well as by exogenous large or unusually large vWF multimers, requires adenosine diphosphate and is resistant to aspirin. Blood 1988; 71: 1366-74.

5. Fujimoto T, Hawiger J. Adenosine diphosphate induces binding of von Willebrand factor to human platelets. Nature 1982; 297: 154-6.

6. Gralnick HR, Williams SB, Coller B. Fibrinogen competes with von Willebrand factor for binding to the glycoprotein IIb /IIIa complex when platelets are stimulated with thrombin. Blood 1984; 64: 797-800.

7. Scott, JP, Montgomery RR, Retzinger GS. Dimeric ristocetin flocculates proteins, binds to platelets, and mediates von-Willebrand factor-dependent agglutination of

platelets. J Biol Chem 1991; 266: 8149-55.

8. Goto S, Salomon DR, Ikeda Y, Ruggeri ZM. Characterization of the unique mechanism mediating the shear-dependent binding of soluble von Willebrand factor to platelets. J Biol Chem 1995; 270: 23352-61.

9. Ruggeri ZM, De Marco L, Gatti L, Bader R, Montgomery RR. Platelets have more than one binding site for von Willebrand factor. J Clin Invest 1983; 72: 1-12.

10. Taylor AD, Neelamegham S, Hellums JD, Smith CW, Simon SI. Molecular dynamics of the transition from L-selectin to $ß_2$-integrin dependent neutrophil adhesion under defined hydrodynamic shear. Biophys J 1996; 71: 3488-500.

11. 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.

12. Goldsmith HL, Frojmovic MM, Braovac S, McIntosh F, Wong,T. Adenosine diphosphate-induced aggregation of human platelets in flow through tubes. III. Shear and extrinsic fibrinogen-dependent effects. Thromb Haemost 1994; 71: 78-90.

13. Goto S, Ikeda Y, Salvidar E, Ruggeri ZM. Distinct mechanisms of platelet aggregation as a consequence of different shearing flow conditions. J Clin Invest 1998; 101: 479-86.

14. Plow EF, Pierschbacher MD, Ruoslahti E, Marguerie GA, Ginsberg MH. The effect of Arg-Gly-Asp-containing peptides on fibrinogen and von Willebrand factor binding to platelets. Proc Nat Acad Sci USA 1985; 82: 8057-61.

15. Kouns WC, Kirchofer D, Hadváry P, Edenhofer A, Weller T, Pfenninger G, Baumgartner HR, Jennings LK, Steiner B. Reversible conformational changes induced in Glycoprotein GP IIb- IIIa by a potent and selective peptidomimetic inhibitor. Blood 1992; 10: 2539-47.

16. Schor K, Darius H, Matzky R, Ohlendort R. The antiplatelet and cardiovascular actions of a new carbaxylic derivative (ZK 36374)- equipotent to PGI_2 in vitro. Arch Pharm 1981; 316: 252-5.

17. Coller BS, Peerschke EI, Scudder LE, Sullivan CA. Studies with a murine monoclonal antibody that abolishes ristocetin-induced binding of von Willebrand factor to platelets; additional evidence in support of GPIb as a platelet receptor for von Willebrand factor. Blood 1983; 61: 99-110.

18. Coller BS, Peerschke EI, Scudder LE, Sullivan CA. A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins IIb and/or IIIa. J Clin Invest 1983; 72: 325-38.

19. Shattil SJ, Cunningham M, Hoaxie JA. Detection of activation platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. Blood 1987; 70: 307-15.

20. Handa M, Titani K, Holland LZ, Roberts JR, Ruggeri ZM. The von Willebrand Factor-binding domain of platelet membrane Glycoprotein GPIb. J Biol Chem 1986; 261: 12579-85.

21. Weinstein M, Vosburgh E, Phillips M, Turner N, Chute-Rose L, Moake J. Isolation

from commercial aurin tricarboxylic acid of the most effective polymeric inhibitors of von Willebrand factor interaction with Glycoprotein Ib. Comparison with other polyanionic and polyaromatic polymers. Blood 1991; 78: 2291-8.

22. Girma JP, Fressinaud E, Christophe O, Roualt C, Obert B, Takahashi Y, Meyer D. Aurin tricarboxylic acid inhibits platelet adhesion to collagen by binding to the 509-695 disulphide loop of von Willebrand factor and competing with Glycoprotein Ib. Thromb Haemost 1992; 68: 707-13.

23. Ruggeri ZM, Zimmerman TS, Russell S, Bader R, deMarco L. Purification of von Willebrand factor. Methods in Enzymology 1992; 215: 265-8.

24. Ruggeri ZM, Zimmerman TS. The complex multimeric composition of factor VIII/von Willebrand factor. Blood 1981; 57:1140-3.

25. Zaleski A, Henriksen RA. Visualization of the multimeric structure of von Willebrand factor using a peroxidase-conjugated second antibody. J Lab Clin Med 1986; 107: 172-5.

26. Tang SS, Frojmovic MM. The effects of pCO_2 and pH on platelet shape change and aggregation for human and rabbit platelet-rich plasma. Thromb.Res. 1977; 10: 135-45.

27. The TH, Feltkamp TEW. Conjugation of fluorescein isothiocyanate to antibodies. I. Experiments on the conditions of conjugation. Immunol 1970;18: 865-73.

28. Xia Z, Wong T, Liu Q, Kasirer-Friede A, Brown E, Frojmovic MM. Optimally functional fluorescein isothiocyanate-labelled fibrinogen for quantitative studies of binding to activated platelets and platelet aggregation. Br J Haematol 1996; 93: 204-14.

29. Williams SB, McKeown LP, Krutzch H, Hansmann, Gralnick H. Purification and characterization of human platelet von Willebrand factor. Br J Haematol 1994; 88: 582-

91.

30. Frojmovic MM, Mooney RF, Wong T. Dynamics of platelet glycoprotein IIb-IIIa receptor expression and fibrinogen binding. I. Quantal activation of platelet subpopulations varies with adenosine diphosphate concentration. Biophys J 1994; 67: 2060-8.

31. Xia A, Frojmovic MM. Aggregation efficiency of activated normal or fixed platelets in a simple shear field: Effect of shear and fibrinogen occupancy. Biophys J 1994; 66: 2190-201.

32. Smoluchowski M von. Versuch einer mathematischen Theorie der Koagulationskinetik kolloider Lösungen. Z Phys Chem 1917; 92: 129-68.

33. Harrison RL, McKee PA. Comparison of thrombin and ristocetin in the interaction between von Willebrand factor and platelets. Blood 1983; 62: 346-53.

34. Azzam K, Cissé-Thiam M, Drouet L. The antithrombotic effect of aurin tricarboxylic acid in the guinea pig is not solely due to the interaction with the von Willebrand factor-GPIb axis. Thromb Haemost 1996; 75: 203-10.

35. Greco NJ, Jones GD, Tandon NN, Kornhauser R, Jackson B, Jamieson GA. Differentiation of the two forms of GPIb functioning as receptors for alpha-thrombin and von Willebrand factor: Ca^{2+} responses of protease-treated human platelets activated with alpha thrombin and the tethered ligand peptide. Biochem 1996; 35: 915-21.

36. Siedlicki CA, Lestini BJ, Kottke-Marchant K, Eppell SJ, Wilson DL, Marchant RE. Shear-dependent changes in the three-dimensional structure of human von Willebrand factor. Blood 1996; 88: 2939-50.

37. Lawrence MB, Springer TA. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion to integrins. Cell 1991; 65: 859-73.

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

39. Ozaki Y, Satoh K, Yatomi Y, Miura S, Fujimura Y, Kume S. Protein tyrosine phosphorylation in human platelets induced by interaction between glycoprotein Ib and von Willebrand factor. Biochim Biophys Act 1995; 1243: 482-8.

40. Chediak J, Telfer C, Laan BV, Maxey B, Cohen I. Cycles of agglutination-disagglutination induced by ristocetin in thrombasthenic platelets. Br J Haematol 1979; 43: 113-26.

41. Manley, R. St. J., and S.G. Mason. Particle motions in sheared suspensions. II. Collisions of uniform spheres. J. Colloid Sci 1952; 7: 354-69.

42. Greco NJ, Tenner Jr. TE, Tandon NN, Jamieson GA. PPACK-Thrombin inhibits thrombin-induced platelet aggregation and cytoplasmic acidification, but does not inhibit platelet shape change. Blood 1990; 75: 1983-90.

43. Bell DN, Spain S, Goldsmith HL. The ADP-induced aggregation of human platelets in flow through tubes: I. Measurement of the concentration and size of single platelets and aggregates. Biophys J 1989; 56: 817-28.

44. Bell DN, Spain S, Goldsmith HL. The ADP-induced aggregation of human platelets in flow through tubes: II. Effect of shear rate, donor sex, and ADP concentration. Biophys

J 1989; 56: 829-43.

45. Frojmovic MM, Mooney RF, Wong T. Dynamics of platelet glycoprotein Iib-IIIa expression and fibrinogen binding. II. Quantal activation parallels platelet capture in stir-associated microaggregation. Biophys J 1994; 67: 2069-75.

46. Suzuki H, Kinlough-Rathbone RL, Packham MA, Tanoue K, Yamazaki H, Fraser M. Immunocytochemical localization of fibrinogen during thrombin-induced aggregation of washed human platelets. Blood 1988; 71: 1310-20.

47. Heilmann E, Hourdille P, Provost A, Papponneau A, Nurden AT. Thrombin-induced platelet aggregates have a dynamic structure. Arterioscl and Thrombos 1991; 11: 704-18.

48. Dong JF, Hyun W, Lopez JA. Aggregation of mammalian cells expressing the platelet glycoprotein (GP) Ib-IX complex and the requirement for tyrosine sulfation of GP Ib alpha. Blood 1995; 86: 4175-83.

49. Berger G, Massé JM, Cramer EM. Alpha-granule membrane mirrors the platelet plasma membrane and contains the glycoproteins Ib, IX and V. Blood 1996; 87:1385-95.

50. Parker RI, Shafer BC, Gralnick HR. Platelet density-dependent partition of platelet von Willebrand factor between alpha granule and non-alpha granule pools. Thromb Haemos 1987; 58: 911-4.

51. Parker RI, Rick ME, Gralnick HR. Effect of calcium on the availability of platelet von Willebrand factor. J Lab Clin Med 1985; 106: 336-42.

52. Harrison P, Cramer EM. Platelet α- granules. Blood Reviews 1993; 7: 52-62.

Introduction to Chapter 4

Thrombosis is a major problem in industrialized nations. Because of the central role of thrombin in cardiovascular ailments, much effort has gone into the development of antagonists to neutralize thrombin's activity. Two such compounds are hirudin and D-Phe-Pro-Arg-chloromethyl ketone (PPACK) which have been assessed in clinical trials. However, due to bleeding complications in several patients, these trials were halted prematurely. These antagonists also counter the occupancy of platelet thrombin receptors by catalytically functional thrombin. Previous studies have suggested, that occupancy is only important initially for inducing normal secretion and consequently, platelet aggregation in flow. We however probe residual effects that these antagonists may have on platelet aggregation, and the surface expression of granule proteins TSP, P-selectin and GPIIb-IIIa receptor activation. Particular attention was paid to antagonist effects on vWF (soluble or surface-expressed)-platelet interactions, due to our previously demonstrated important role for vWF in shear-associated aggregation, crucial for the arrest of hemorrhage. These parameters are examined at different thrombin concentrations.

Chapter 4

Thrombin Receptor Occupancy Modulates Aggregation Efficiency and Platelet Surface Expression of von Willebrand Factor and Thrombospondin, at Low Thrombin Concentrations

Abstract

Previous studies evaluating requirements for occupancy of thrombin receptors in normal platelet secretion and aggregation, using the thrombin antagonists hirudin and PPACK (D-Phe-Pro-Arg-chloromethylketone), have suggested that at low thrombin activating concentrations (0.025 - 0.13 U/ml), occupancy was required only in the first 45 - 60 seconds following activation. In our study, we differentiate between thrombin receptor occupancy requirements for surface expression of secreted adhesive proteins, for activation of GPIIb-IIIa receptors, and for aggregation of washed platelets (WP) in laminar shear flow. Platelets activated with 0.05 U/ml thrombin for 10 minutes to allow maximal secretion (hereafter referred to as "pre-activated platelets"), then sheared, showed a 50 - 70 % decrease in platelet counts after 60 s of shear. Treatment of pre-activated platelets with hirudin or PPACK produced a 65 % reduction of capture efficiencies, α_G (reflecting experimental/theoretical initial rates of aggregation), as well as a 30-40 % decrease in the surface expression of von Willebrand factor (vWF) and thrombospondin (TSP). However, α-granule membrane P-selectin expression and numbers of activated GPIIb-IIIa receptors were comparable for treated and non-treated platelets. No significant difference in any of the parameters tested was observed when platelets were similarly pre-activated with 0.2 U/ml thrombin, due to treatment with thrombin antagonists. Binding of soluble FITC-vWF (GRGDSP-sensitive) to pre-activated, thrombin antagonist treated platelets, was greatly reduced (≥80 %). Soluble Fg was shown to bind to antagonist-treated pre-activated platelets, but could not significantly enhance platelet aggregation. Although occupancy of thrombin receptors by catalytically active thrombin is required transiently for secretion and activation of platelets, there is a further requirement for thrombin occupancy at low thrombin concentrations, for optimizing initial rates of platelet aggregation, surface expression of vWF and TSP, and activated GPIIb-IIIa ligand recognition.

1. INTRODUCTION

Hirudin and PPACK (D-Phe-Pro-Arg-chloromethyl-ketone) are direct thrombin antagonists; hirudin occupies both the anion binding exosite involved in fibrinogen (Fg) recognition (1)), and the catalytic site (2) of thrombin, and blocks binding to platelets (3), while PPACK directly inactivates the catalytic site while still permitting binding of thrombin to platelets through the exosites, but not supporting aggregation (4,5). Hirudin and hirulog (a thrombin antagonist consisting of a thrombin exosite recognition domain linked by glycyl residues to a PPACK analog) have been evaluated in clinical trials as anti-thrombotics drugs (6-8). Although some reduction in the incidence of thrombotic episodes was reported, several of these trials were stopped prematurely due to bleeding complications in some patients (9). These bleeding complications may result from decreased platelet aggregation, a prerequisite for clot formation, or from diminished or impaired Fg cleavage, due to a decrease in the quantity or quality of available thrombin.

Thrombin antagonists have previously been used to show a requirement for thrombin occupancy of platelet receptors for secretion and aggregation (investigated in the stirred vials of an aggregometer at thrombin activating concentrations between 0.025 - 0.12 U/ml), in the first 45 - 60 seconds following thrombin activation, but not once aggregation had begun (3, 10,11). Thus, a time-limited requirement for thrombin occupancy for secretion and aggregation was suggested. However, we hypothesize that hirudin and PPACK, by limiting available functional thrombin (i.e. catalytically-active thrombin), may have more prolonged effects on platelet function, primarily when platelets are activated with low thrombin concentrations, where activation is expected to be mediated by thrombin binding

to the GPIb receptor (K_d = 0.03 - 0.06 U/ml (0.3 - 0.6 nM)) (12). Hence, it is important to investigate the effect of these antagonists on platelet aggregation at subnanomolar thrombin concentrations and under well-controlled physiological shear rates. Accordingly, we set out to examine the time-dependent effect of hirudin and PPACK on the activation of platelets at low thrombin concentrations (0.05 U/ml), as reflected by surface expression of secreted adhesive proteins and upregulation of GPIIb-IIIa receptor binding capacity, as well as examining the effect of these antagonists on aggregation at well-defined shear rates. These studies were contrasted with experiments using higher thrombin concentrations (0.2 - 1 U/ml), associated with a distinct thrombin receptor (K_d = 0.2 - 0.5 U/ml;(12)).We were thus able to differentiate between requirements for functional thrombin occupancy of thrombin-induced platelet activation, versus platelet aggregation.

2. MATERIALS AND METHODS

2.1. Reagents:

Ristocetin A grade, ADP, α-thrombin and hirudin were purchased from Sigma Corp. USA; glutaraldehyde, electron microscopy grade, from Polysciences Inc.; paraformaldehyde from Fisher Scientific Co., Fairlawn, NJ; human fibrinogen from Enzyme Research Laboratories Inc., South Bend, IN; H-Gly-Arg-Gly-Asp-Ser-Pro-OH peptide (GRGDSP) peptide, used to block the binding pocket of GPIIb-IIIa (13); and PPACK(14) (D-Phe-Pro-Arg chloromethylketone HCl) from Calbiochem Corporation, La Jolla, CA; fluorescein-isothiocyanate (FITC; Isomer I) from Boehringer Diagnostics, La Jolla, CA); ZK 36374, a

stable prostacyclin analog (15), was a gift from Dr. T. Krais, Schering Co., Berlin, Germany. Monoclonal antibody (MAb) 6D1 an IgG against the vWF binding site on GPIb (16) was kindly donated by Dr. B. Coller, State University of New York at Stony Brook; MAb S.12, an IgG1, directed against platelet membrane CD62-P, PADGEM-140 (17), was a gift from Dr. McEver, Oklahoma Medical Research Foundation, Oklahoma City, OK; MAb 2.2.9., an IgG1, recognizing an epitope, non-interfering with vWF binding to platelets, between residues 1366-2050 of vWF (18) was generously provided by Dr. Z. Ruggeri, Scripps Institute, La Jolla, CA; MAb 5G11, an IgG2a recognizing a non-functional epitope on thrombospondin (TSP)(19) was prepared in one of our labs: Dr. Chantal Legrand, Hôpital St. Louis, Paris, France; Mab FITC-PAC-1, which recognizes an activation-dependent epitope on GPIIb-IIIa (20), was purchased from Becton-Dickinson, San Jose, CA. Calibration beads, Quikcal: 7.1 μm, used for fluorescence intensity calibration of FITC-labelled proteins, were obtained from Flow Cytometry Standards Corp, Research Triangle Park, NC. Latex beads used for adsorbing GPIIb-IIIa were purchased from Interfacial Dynamics Corp, Portland, OR.

2.2. Purification of von Willebrand factor and GPIIb-IIIa: von Willebrand factor was prepared from human cryoprecipitate obtained from the Canadian Red Cross and purified as previously described (21). GPIIb-IIIa was isolated from human platelet membranes by lentil lectin affinity chromatography followed by gel filtration chromatography (22) and elution from a Sephacryl gel filtration column with an HSC buffer (in mM: HEPES 5, NaCl 150, $CaCl_2$ 3, pH 7.4) containing 30 mM of β-OG (n-octyl-β-D-glucopyranoside)-solubilized

GPIIb-IIIa.

2.3. Preparation of washed platelets: Washed platelets (WP) were prepared by a one-step wash procedure from human citrated whole blood as previously described (23), involving acidification of platelet rich plasma (PRP) to pH 6.5 and by addition of 50 nM ZK 36374, a synthetic prostacyclin analog, to inhibit platelet activation. The remaining pellet after centrifugation at 800xg for 15 minutes, was resuspended in half the original volume, in a modified Tyrode buffer (BAT:136 mM NaCl, 2.7mM KCl, 11.9 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 1.0 mM $MgCl_2$, 5.6 mM glucose, 0.35% BSA). Platelet suspensions were kept at 37° C under a mixture of 95% air and 5% CO_2 to maintain pH 7.4 (24).

2.4. Preparation of GPIIb-IIIa* latex beads: Aldehyde/Sulfate Polystyrene latex beads, 4.5 μm, were washed with Incubation Buffer (IB; Tyrode pH 6.5, 100 mM Hepes), then suspended for 2 hours at room temperature in IB containing 25 μg/ml purified GPIIb-IIIa and 1 mM GRGDSP to activate the GPIIb-IIIa complex (GPIIb -IIIa*). Beads were washed and resuspended in BSA buffer (Tyrode pH 7.4. 10 mM Hepes, 2.5% BSA) and left overnight at 4^0 C, followed by a further wash with storage buffer (Tyrode pH 7.4, 3.6 mM Hepes).

2.5. Labelling of vWF and monoclonal antibodies: vWF, Fg and antibodies were labelled with fluorescein-isothiocyanate, and fluorescein to protein ratios (F:P) were calculated as previously described (21).

2.6. Binding experiments:

2.6.1. Ristocetin or thrombin-mediated binding of FITC-vWF to washed platelets: Washed platelets (WP) ($10^4/\mu l$), in BAT buffer, containing freshly-added 1 mM $CaCl_2$, were incubated with either 0.5, 0.8 or 1.2 mg/ml ristocetin and 10 μg/ml FITC-vWF in the presence or absence of hirudin, or with 0.05 U/ml thrombin and FITC-vWF at concentrations between 1-35 μg/ml for 25 minutes, to allow equilibrium binding. Specificity of binding to GPIIb-IIIa* was determined by incubating thrombin-activated WP with FITC-vWF in the presence of 1 mM GRGDSP. For experiments with thrombin antagonists, platelets were allowed to undergo activation by thrombin for 10 minutes (hereafter referred to as "pre-activated" platelets), to correspond to incubation times used for aggregation experiments, then were treated with an 8-fold excess of hirudin (previously reported to neutralize thrombin at a 4 to 40-fold excess ; (10,25-27)) or with 2 μM PPACK (11,28), for 2 minutes, as neutralization is known to occur rapidly (29). Soluble FITC-vWF was then added, and incubated for a further 25 minutes. The reaction was arrested by diluting platelets with 10 volumes of BAT buffer and the average fluorescence per platelet particle read immediately on a flow cytometer, FACScan (Becton Dickinson, Mississauga, Ont), as previously described (30,21). Platelets were identified on the basis of characteristic FSC/SSC profiles, and gates were set to exclude aggregates from platelet populations used to generate fluorescence histograms. Particular attention was paid to the bimodal distribution of fluorescence due to resting (P^0) and activated platelets (P^*), showing fluorescence greater than control values in the absence of activator, as previously described (30). Thus, the fraction of cells activated is reported as % P^* for bimodal, subpopulation responses, or

simply as % activated platelets for partial, unimodal responses, where the entire population undergoes a partial activation. Accordingly, fluorescence for the total cell population is reported as Fl_{tot}, while FL^* represents the fluorescence on the activated platelet population (P^*) only.

2.6.2. Effect of hirudin and PPACK on thrombin-induced platelet secretion and surface-bound protein: In order to assess the effects of the thrombin antagonists on platelet activation, WP were activated with thrombin either in the absence or presence of hirudin and PPACK. MAbs FITC-2.2.9, FITC-5G11, FITC-S.12 were used to report on the amount of surface-expressed vWF (SE-vWF), TSP (SE-TSP), and P-selectin respectively. Five μl of WP, at 4 x 10^4 /μl in BAT buffer, were added to FACS tubes with 0.05 U/ml or 0.2 U/ml thrombin and incubated for 30 minutes in a total volume of 20μl, containing buffer, 1mM Ca^{2+} and either 400 nM FITC 2.2.9, 333 nM FITC-5G11 or 273 nM FITC-S12. In experiments with thrombin antagonist, either hirudin at an 8-fold excess concentration, or 2μM PPACK was added immediately prior to thrombin, or to platelets pre-activated with thrombin, and incubated for 2 minutes. Mabs were subsequently added and incubated for a further 30 minutes. Platelet suspensions were diluted for reading on the FACScan as described above. The binding of reporting antibody to WP was expressed as the percentage inhibition of control values, obtained in the absence of antagonist.

2.6.3. Effect of hirudin on thrombin-induced glycoprotein receptor GPIIb-IIIa activation: FITC-PAC-1 was used to report on activated platelet GPIIb-IIIa receptors (GPIIb-IIIa*). WP (10^4 /μl) were either activated with thrombin and incubated together with

333 nM FITC-PAC-1, or pre-activated, treated with an 8-fold excess of hirudin, then incubated for a further 30 minutes with FITC-PAC-1. Platelet suspensions were diluted for reading on the FACScan as described above.

2.6.4. Percent Fg occupancy of GPIIb-IIIa*: Since there is an ideal range of Fg occupancies of available GPIIb-IIIa* receptors which is expected to yield optimal capture efficiencies (approximately 20-75%; (31)), we adjusted Fg occupancies on platelets used for shearing to fall within this range. Accordingly, WP ($10^4/\mu l$) were pre-activated with 0.05 U/ml thrombin, incubated with hirudin or PPACK for 2 minutes, then incubated for a further 5 - 8 minutes with 0.2 μM FITC-Fg . The reaction was arrested by diluting with 10 volumes BAT buffer, and results were read immediately on the FACScan. Alternatively, incubations were for 40 minutes with saturating concentrations of Fg, to determine maximal numbers of available activated GPIIb-IIIa receptors. Numbers of FITC-Fg bound were determined from average fluorescence values, using MESF Quikcal calibration beads, from the equation: 220(fluorescence) - 1099/ F:P, as previously reported (32). Thus, the percent occupancy of Fg was estimated by: # Fg bound at 5 minutes/ # Fg receptors available at 40 minutes.

2.6.5. Binding to GPIIb-IIIa* latex beads: FITC-vWF (1-35 μg/ml) was incubated for 30 minutes with latex beads with adsorbed activated GPIIb-IIIa , in BAT buffer solution, or in BAT containing 0.5 U/ml hirudin or 2 μM PPACK. Bound vWF was determined from fluorescence measurements, as described above.

2.7. Aggregation in shear:

Aggregation of washed platelets under uniform laminar shear was tested in a coaxial cylinder microcouette device (0.3 mm gap) at shear rates from 300 - 1000 s^{-1}, as previously described (21,31). Samples were drawn at given time intervals after the onset of shear (0, 10, 20, 60 sec), immediately fixed in 10 vol of 0.2% glutaraldehyde, and further diluted 7-fold with isotonic saline, for analysis of particle number with the FACScan. Platelet aggregation (PA) was calculated by determining the decrease in particle number with time: % PA= (1 - N_t/N_0) x 100%, where N_t = platelet particle number at time t, and N_0 = the initial platelet number. Capture efficiencies α_G, defined as the ratio of the rate of two-body collisions resulting in aggregate formation to the total rate of two-body collisions, were computed based on equations by Smoluchowski, assuming dilute suspensions of rigid spheres (33), as described by Xia & Frojmovic (31).

2.7.1. Effect of hirudin and PPACK on shear-associated aggregation of WP activated by thrombin or ADP: Washed platelet suspensions (4 x $10^4/\mu l$) were pre-mixed in Eppendorf tubes with BAT buffer containing 1mM Ca^{2+}, and incubated with thrombin for 10 minutes (determined to yield optimal capture efficiencies relative to similarly activated platelets incubated for shorter periods of time; results not shown) prior to transfer to the microcouette for shearing. Initially platelets were activated with three different concentrations of thrombin, 0.05, 0.2 and 1.0 U/ml, respectively chosen to represent thrombin binding to the high affinity binding site on GPIb, an intermediate concentration, and one at which platelet responses would be mediated through the thrombin seven-

transmembrane receptor (PAR-1; (12)). For hirudin and PPACK experiments, platelets were pre-incubated with thrombin in the presence of the antagonists, or else were pre-activated, treated with hirudin or PPACK for 2 minutes, and immediately transferred to the microcouette for shear experiments. In experiments examining the effect of the length of the pre-incubation time on thrombin antagonist inhibition of platelet aggregation, platelets were pre-incubated with thrombin for 1 minute or 10 minutes. To test the effects of soluble Fg on platelet aggregation, thrombin antagonist-treated platelets were incubated for a further 5-8 min with 0.2 μM Fg, to yield optimal Fg occupancies, prior to shearing.

The effect of hirudin on ADP-induced shear aggregation was also examined. WP were activated with 5 μM ADP in Eppendorf tubes as in previous studies (34) and immediately transferred to the microcouette for shearing, or ADP was added together with 0.5 U/ml hirudin and subsequently sheared as above.

2.7.2. Statistical Analysis

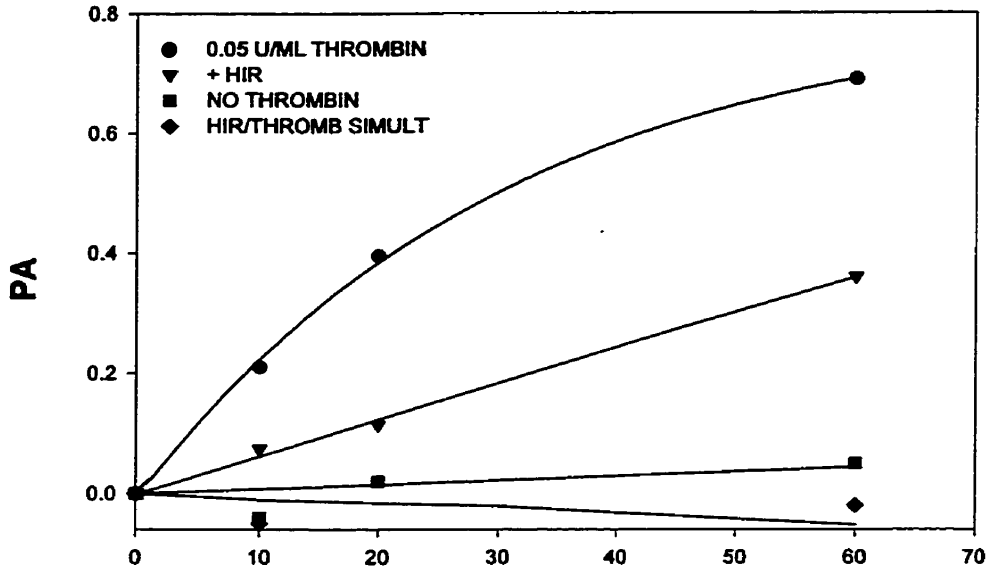
Data are presented as the mean ± standard error of the mean (SEM), with numbers in parentheses indicating the number of experiments performed. Tests for statistical significance were done using the Student t-test.

3. RESULTS

3.1. Aggregation in sheared suspensions

Using our washed platelet preparations (WP) and light transmission aggregometry (detecting macroaggregates of ≥ 10 platelets; (35)), we were able to confirm the earlier results obtained by Holmsen (10) and Greco (11), where hirudin or PPACK inhibited the aggregation of platelets activated with low thrombin concentrations, if added to the stirred WP in the vials of an aggregometer within ≤ 1 minute of activation (results not shown). We then examined the effect of thrombin antagonists on the microaggregation (platelet counts determined by flow cytometry) of platelets sheared in a microcouette device at the physiologic shear rates of G = 300 and 1000 s^{-1}. Washed platelets activated with 0.05 U/ml thrombin in the presence of an 8-fold excess of hirudin or 2 μM PPACK, and sheared at G = 1000 s^{-1}, did not aggregate, showing that the "neutralized" thrombin could not yield any detectable microaggregation in shear flow. Alternatively, platelets were incubated with thrombin for times exceeding the previously-observed lag phase of ≤1 minute (10,11). Platelets activated with 0.05, 0.2 or 1.0 U/ml thrombin were maintained under non-flow conditions for 10 minutes (referred to as "pre-activated" platelets), and then subjected to a uniform shear field of G = 1000 s^{-1}. Such pre-activated platelets were found to aggregate in shear flow (Fig.4-1) with equal or even greater rates of initial aggregation than platelets sheared immediately following activation. Since it was found that the extent of aggregation and its inhibition by antagonists was equivalent when platelets were pre-activated with 0.2 or 1.0 U/ml thrombin (results not shown), subsequent experiments were carried out at 0.05

a.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+ HIR
NO THROMBIN
HIR/THROMB SIMULT
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50
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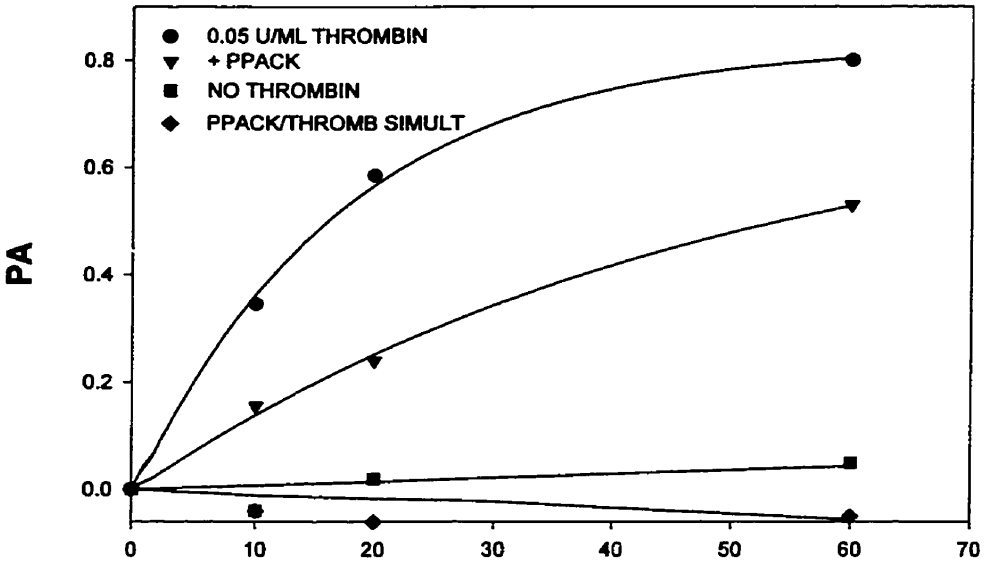
70

b.


0.05 U/ML THROMBIN
+ PPACK
NO THROMBIN
PPACK/THROMB SIMULT
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c.

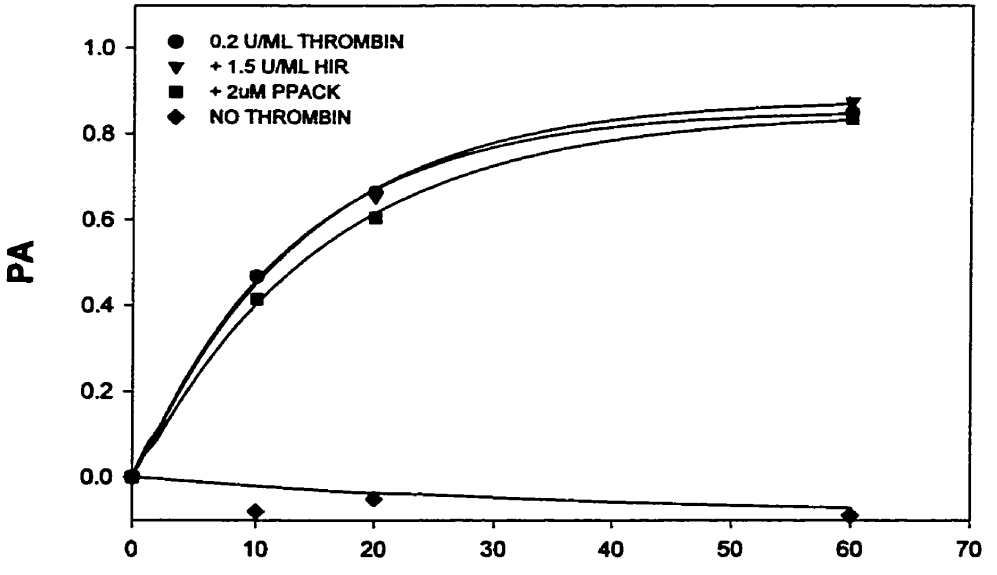
0.2 U/ML THROMBIN
+ 1.5 U/ML HIR
+ 2uM PPACK
NO THROMBIN
PA
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
50
60
70
TIME(SEC)

Fig. 4-1. Kinetics of shear aggregation. Washed platelets (WP) at 4 x 10^4 /μl were activated with 0.05 or 0.2 U/ml thrombin and buffer, or with the antagonists hirudin or PPACK, added either together with thrombin and incubated for 10 minutes (shown as HIR/THROMB SIMULT, and PPACK/THROMB SIMULT) or after 10 minutes of incubation with thrombin ("pre-activated"). Platelet suspensions were transferred 2 minutes after addition of antagonist to a microcouette device to evaluate platelet aggregation (PA) under shear flow. Results shown are for shear aggregation at G = 1000 s^{-1} for a representative donor. *a.*0.05 U/ ml thrombin, treatment with hirudin; *b.*0.05 U/ml thrombin, treatment with PPACK; *c.* 0.2 U/ml thrombin.

or 0.2 U/ml only.

The effect of hirudin and PPACK on the shear-associated aggregation of platelets pre-activated with thrombin was then investigated. Analysis of kinetic PA curves for α_G showed that the α_G for 0.2 U/ml thrombin-induced aggregation did not change significantly following treatment of pre-activated platelets with thrombin antagonist (Table 4-1), even when a 20-fold excess of hirudin was used. However, for similar studies with 0.05 U/ml thrombin, there was a significant decrease in α_G (Fig.4-2), with an accompanying decrease in the extent of aggregation at 60 seconds, whether platelets were pretreated for 10 minutes (Fig. 4-1; Table 4-1), or for 1 minute (results not shown) prior to treatment with antagonist. The thrombin antagonist effects on platelet aggregation are specific to low thrombin concentration-mediated aggregation, as shown by the lack of significant difference in the shear aggregation of WP activated with a sub-maximal concentration of ADP (5 μM) in the presence of 0.5 U/ml hirudin (Fig. 4-2).

Table 4-1. Summary of inhibition by hirudin and PPACK, of parameters of thrombin activation

		% INHIBITION FOLLOWING HIR/PPACK NEUTRALIZATION FOR PLATELETS PRE-ACTIVATED WITH:			
Parameter		Low Thrombin (0.05U/ml)		HighThrombin (0.2U/ml)	
		Hirudin	PPACK	Hirudin	PPACK
[a]Aggregation at $G=1000\ s^{-1}$	α_G	[b,c] 64 ± 5%*	60 ± 9%*	-14 ± 12	-6 ± 5
	PA_{60s}	48 ± 7%*	43 ± 7%*	0.3 ± 3	-3.7 ± 4
		[d](12)	(6)	(3)	(3)
Surface-bound[e] :	P_s	3.5 ± 8%	-10 %	2.5 %	1.5%
		(4)	(2)	(2)	(2)
	vWF	41 ± 6%*	16 %**	-10 ±14 %	18%
		(12)	(2)	(5)	(2)
	TSP	41 ± 4%*	29 ±12%*	14 ± 8%	12%
		(6)	(3)	(5)	(2)

[a] Note that results for α_G ,PA_{60s} and surface-expressed proteins are for platelets pre-activated with thrombin for 10 minutes.

[b] Note that α_G values were essentially identical for low and high thrombin, 0.09 ±.02 and 0.12 ± .02 respectively, in the absence of any thrombin inhibitors.

[c] Note that inhibition observed for only 1 minute pre-activation of WP with 0.05 U/ml thrombin, prior to addition of hirudin, was comparable to neutralization after a 10 minute pre-activation.

[d] Numbers in parentheses represent numbers of separate experiments

[e] Measured from changes in Fl_{tot}

* $p < 0.001$; ** $p < 0.05$

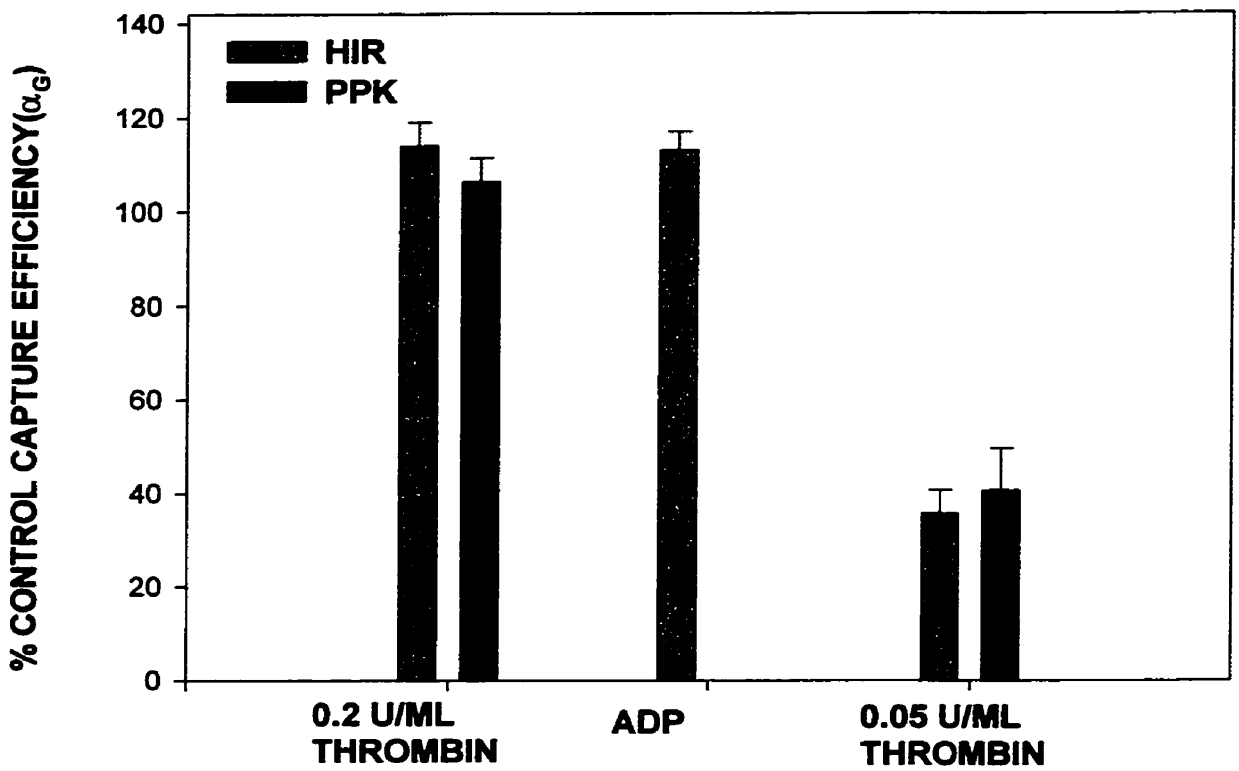

Fig. 4-2. Effect of hirudin or PPACK on capture efficiencies (α_G) for variously-activated platelets in a sheared suspension. For platelets pre-activated with thrombin and treated with antagonist, conditions were as described for Fig.1. Activation with 5 μM ADP was done in the presence of hirudin for 2 minutes, after which platelets were transferred to the microcouette and sheared. Results shown are expressed as % control (no antagonist) $\alpha_G \pm$ SEM.

3.2. Effect of hirudin and PPACK on thrombin-induced secretion: The effect of hirudin and PPACK on platelet secretion was compared for platelets activated with thrombin and treated with antagonist immediately following addition to platelet suspensions, or after a 10 minute pre-incubation, at which time granule secretion is complete (36). Washed platelets were incubated under equilibrium binding conditions with thrombin and monoclonal antibodies (MAb) FITC-2.2.9 to report on surface-expressed vWF (SE-vWF) or FITC-5G11, reporting on SE-TSP, indicating release of soluble granule contents, or with FITC-S.12 reporting on P-selectin (indicating fusion of α-granule membrane with plasma membrane). The extent of expression of surface-bound proteins (both Fl^* and % P^*) on platelets pre-activated with 0.05 U/ml thrombin at 10 minutes (incubation times used for shear) and 30 minutes (time for equilibrium binding experiments) was compared. Relative expression of SE-vWF was examined by determining on-rates for FITC-2.2.9, and was found to be almost identical for both thrombin pre-incubation times, indicating that maximal expression, both in numbers of vWF and % P^*, had occurred by 10 minutes following thrombin activation. A similar technique was previously used to evaluate the time-dependent expression of activation of GPIIb-IIIa receptors on platelets following stimulation with ADP (30).

Hirudin added together with 0.05 U/ml thrombin resulted in minimal or no P-selectin SE-vWF or SE-TSP expression, and no GPIIb-IIIa activation, when compared to non-antagonist treated platelets. Addition of PPACK simultaneously with thrombin also reduced these parameters by >85 %.

The differences observed between the aggregation of thrombin antagonist-treated platelets pre-activated with 0.05 and those pre-activated with 0.2 U/ml thrombin were

paralleled by differences in secretion and expression of surface-bound adhesive proteins. As shown in Fig. 4-3b, platelets pre-activated with 0.2 U/ml thrombin and then treated with hirudin or PPACK, exhibited amounts of fluorescence for FITC-S.12, FITC-2.2.9. and FITC-5G11 similar to those of platelets not treated with antagonist (controls), summarized in Table 4-1. However, when platelets were pre-activated with 0.05 U/ml thrombin, then treated with hirudin or PPACK (Fig. 4-3a), there was a significant decrease of Fl_{tot} for FITC-2.2.9 and FITC-5G11, reporting on SE-vWF and SE-TSP respectively, but not for FITC-S.12 reporting on membrane P-selectin (Table 4-1).

Changes in Fl_{tot} reflect changes both in % P^*, the fraction of cells activated, and Fl^*, the mean fluorescence of the activated population (P^*) only. Thus, we further analysed the fluorescence specifically on activated platelet populations. Analysis of fluorescence histograms showed that decreases in total fluorescence for bound FITC-2.2.9 on WP pre-activated with 0.05 U/ml thrombin, then treated with an 8-fold excess of hirudin, (% I = 50 ± 3% (n=8)), principally represent a decrease in % P^*, and only a small decrease in Fl^*, as shown in Table 4-2. In contrast to an inhibition primarily of % P^* for FITC-2.2.9 by hirudin, the decrease in Fl_{tot} for FITC-5G11 (% I = 41 ± 5 %, n=5), represents almost an equal decrease in Fl^* and in % P^* (% I = 17 ± 5 %, and 19 ± 3%, n = 5), respectively.

3.3 Correlation between capture efficiency and surface-expressed vWF on WP activated with 0.05 U/ml thrombin and treated with antagonist after 10 minutes: Since separate studies showed decreased aggregation and surface-expression of two α-granule proteins when activating with the lower thrombin concentration (results summarized in Table

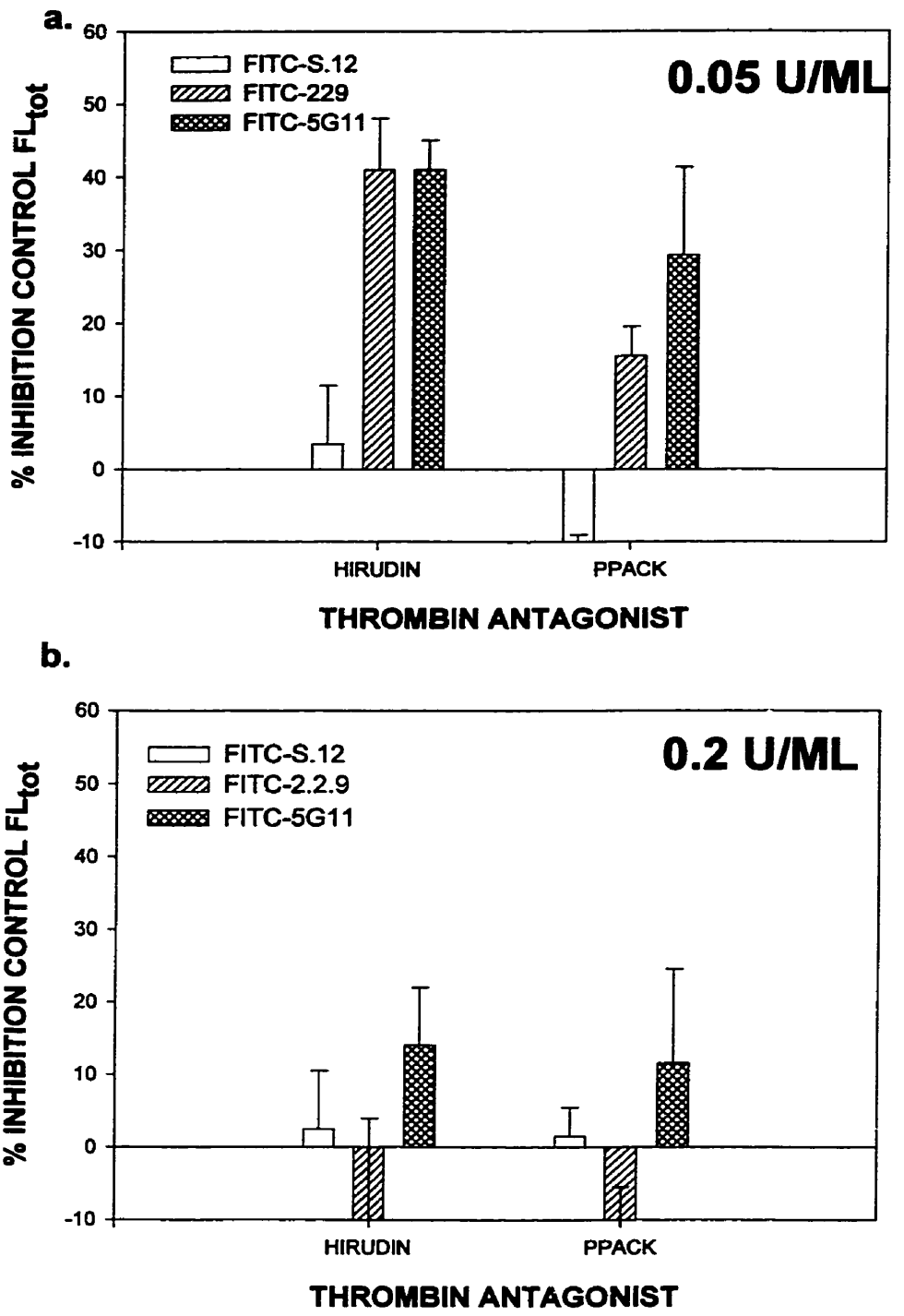

Fig. 4-3. Effect of hirudin or PPACK on the surface expression of P-selectin, vWF and TSP on platelets pre-activated with thrombin, then treated with antagonist. WP (1 x $10^4/\mu$l) were activated with *a.* 0.05 U/ml thrombin or *b.* 0.2 U/ml thrombin. Reporting mAbs against α-granule membrane P-selectin (S.12), vWF (2.2.9) or TSP (5G11) were incubated together with thrombin-activated platelets for 30 minutes, or pre-activated platelets were incubated for 2 minutes with hirudin or PPACK, then incubated for an additional 30 minutes with reporting antibody. Results are expressed as % inhibition of control (no antagonist) fluorescence values ± SEM, for a minimum of three donors, done in duplicate.

4-1), we investigated the relationship between surface-expressed vWF (SE-vWF) and α_G using platelets from the same preparation, in order to minimize inter-donor variability. For platelets pre-activated with 0.05 U/ml thrombin, then antagonist treated, a paired t-test showed a correlation between the percent inhibition (% I) for FITC-2.2.9 for FL* and α_G ($p<0.05$), and % P* and α_G*** ($p< 0.01$) (Table 4-2). However, no significant differences in α_G , SE-vWF or SE-TSP compared to untreated platelets were observed when platelets were pre-activated with 0.2 U/ml thrombin, then treated with antagonist (Table 4-1).

Table 4-2. Relation between the effect of hirudin on capture efficiency (α_G), and the surface expression of vWF on platelets pre-activated with 0.05 U/ml thrombin, and treated with antagonist after 10 minutes

	PERCENT INHIBITION (% I) OF			
DONOR	α_G	%P* [a]	Fl* [b]	Fl_{tot} [c]
1	37	28	24	44
2	77	37	21	49
3	46	47	27	40
4	45	22	17	35
5	83	58	23	57
6	67	47	19	57
7	38	51	21	62
8	66	53	19	58
X±SEM	**58 ± 5**	**43 ± 4****	**21 ± 1*****	**50 ± 3**

[a] fraction of cells activated; %P* = 41 - 71 % in absence of antagonist
[b] fluorescence for activated platelets; Fl* = 175 - 232 in absence of antagonist
[c] fluorescence for the total cell population; Fl_{tot} = 76 - 182 in absence of antagonist
**paired t-test between α_G and % P*, $p< 0.01$
***paired t-test between α_G and Fl* , $p < 0.05$

3.4. Inhibition of soluble vWF binding to thrombin-activated WP by hirudin or PPACK: Since we had observed a decrease in the surface expression of platelet vWF in thrombin antagonist pre-treated platelets, we wanted to determine whether there was a defect in the binding of plasma-derived vWF as well. The binding of FITC-vWF (1 and 35 μg/ml) to WP pre-activated with 0.05 U/ml thrombin, or to pre-activated thrombin antagonist treated platelets, was compared. No change in vWF binding due to the delayed addition of FITC-vWF was observed in the absence of inhibitor. However, hirudin and PPACK both greatly reduced the binding of all concentrations of FITC-vWF to platelets activated with thrombin, with an inhibition by hirudin of 82* ± 3%, and by PPACK, of 79* ± 2% (n = 3); * $p < 0.01$ (Fig. 4-4).

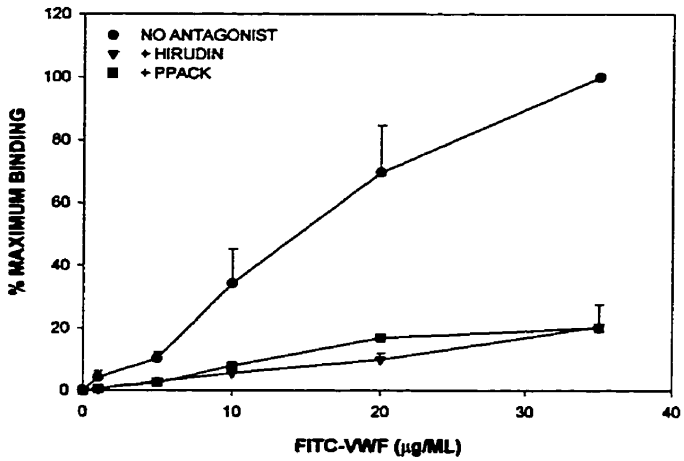

Fig. 4-4. Effect of hirudin and PPACK on the binding of soluble FITC-vWF to thrombin-activated platelets. FITC-vWF was added directly to WP activated with 0.05 U/ml thrombin, or to platelets pre-activated with thrombin, then treated with hirudin or PPACK. In all cases platelet suspensions were allowed to reach equilibrium binding of FITC-vWF. Specific binding was determined by subtracting non-specific fluorescence obtained in the presence of 1mM GRGDSP. Results are presented as the percentage of the maximum vWF bound ● SEM, for each vWF concentration (n = 3).

3.5. Do thrombin antagonists interact directly with soluble vWF? We sought to test whether inhibition of aggregation by hirudin was due specifically to deoccupancy of thrombin from platelets, rather than to a direct interaction with the A-1 domain or RGD site on vWF, both important for cross-bridging of thrombin-activated platelets under shear flow (21). Soluble vWF was incubated with ristocetin, either in the presence or absence of hirudin to determine its effects on vWF-GPIb interactions (Fig. 4-5a), using a range of ristocetin concentrations (0.5 - 1.2 mg/ml) to detect hirudin interference with vWF binding. There was however, no significant change in the ristocetin-mediated binding of FITC-vWF to WP at any ristocetin concentration when platelets were incubated in the presence of hirudin. To test hirudin and PPACK inhibition of vWF-GPIIb-IIIa interactions, soluble FITC-vWF (1 - 35 μg/ml) was incubated with GPIIb-IIIa*-beads in the presence and absence of thrombin antagonists. FITC-VWF bound to the beads (Fig. 4-5b) with a K_d of 30 ± 1 nM (n = 3), which was unchanged in the presence of hirudin or PPACK: K_d = 29 ● 1 nM (n = 3) and 30nM (n = 2), respectively. Thus, the thrombin antagonists do not appear to interfere directly with either vWF- GPIb or vWF- GPIIb-IIIa interactions.

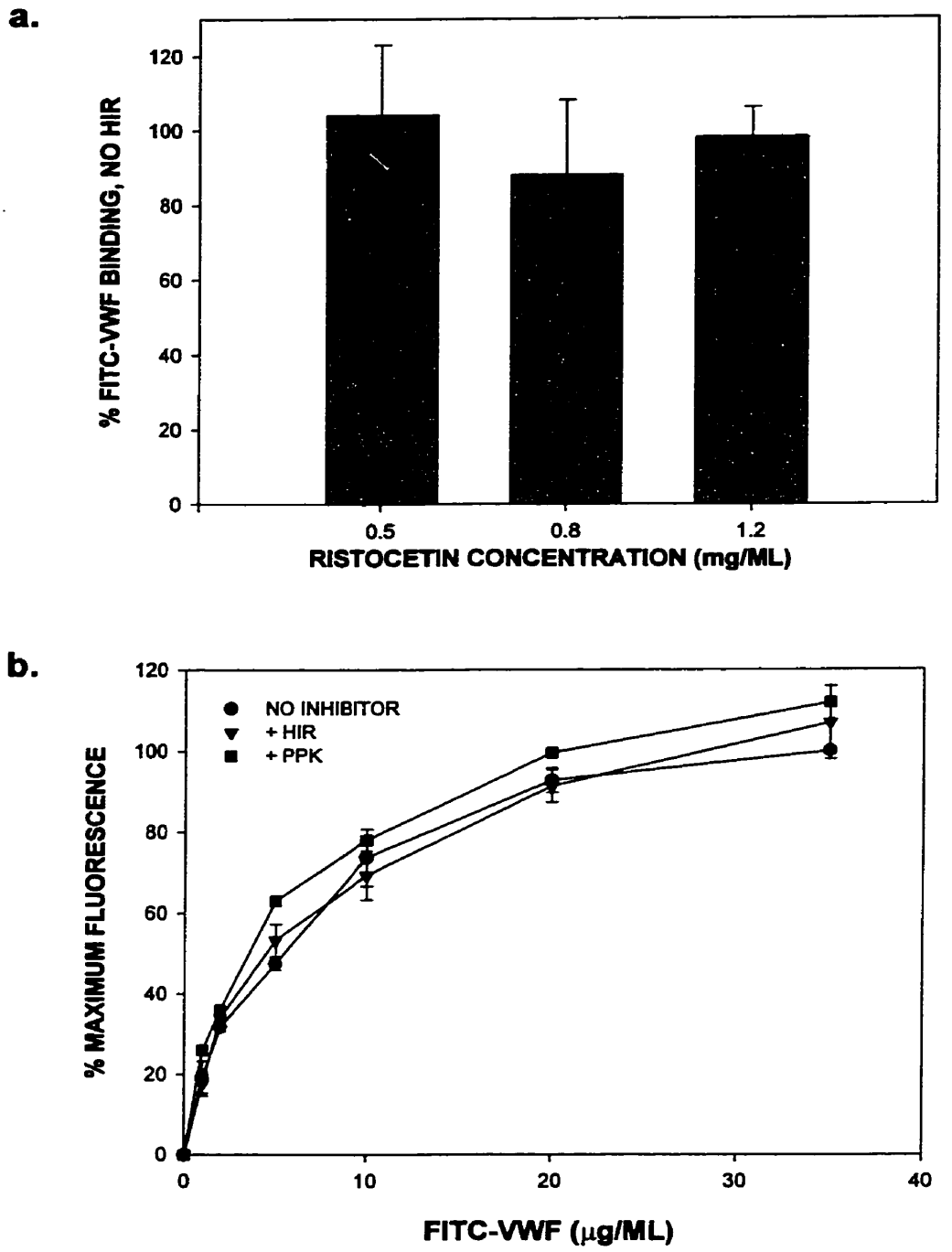

Fig. 4-5. The effect of hirudin and PPACK on the binding of soluble FITC-vWF to either GPIb or activated GPIIb-IIIa. *a.* To test for any direct effect of antagonists on the vWF-GPIb interaction, WP (1 x $10^4/\mu l$) were incubated for 30 minutes with three concentrations of ristocetin and $10\mu g$/ml FITC-vWF, in the presence or absence of hirudin. *b.* Any direct effect on vWF-GPIIb-IIIa interactions was tested in a cell-free system using latex beads with adsorbed activated GPIIb-IIIa (GPIIb-IIIa *). FITC-vWF at varying concentrations was incubated with GPIIb-IIIa*-beads for 30 minutes, and fluorescence was compared to that observed in the presence of hirudin or PPACK. Results are for three experiments ± SEM.

3.6. GPIIb-IIIa activation in the presence of hirudin or PPACK: In contrast to the inhibition of soluble vWF binding to thrombin-activated WP, we have previously shown that platelets pre-activated with thrombin concentrations from 0.02 to 0.5 U/ml, then treated with hirudin, can bind Fg (21). In our present studies, we found that comparable numbers of Fg molecules were bound with 0.5U/ml thrombin or with 0.2 μM PMA, known to produce maximal activation of GPIIb-IIIa receptors (17). Similar results were obtained using PPACK, with maximal numbers of Fg molecules bound showing ≤ 15 % difference from those bound to hirudin treated platelets. To determine whether platelets activated at submaximal thrombin concentrations show altered availability of activated GPIIb-IIIa receptors upon treatment with thrombin antagonists, we used FITC-PAC-1, an IgM monoclonal antibody which recognizes only activated GPIIb-IIIa receptors, thus mimicking Fg-binding, but resistant to direct proteolysis by thrombin. Fluorescence values for FITC-PAC-1 bound to WP pre-activated with thrombin at concentrations from 0.02 to 1.0 U/ml, then treated with hirudin, were compared to fluorescence values for FITC-PAC-1 bound to untreated platelets (Fig.4-6). For platelets pre-activated at low thrombin concentrations ($\leq$ 0.1 U/ml), then treated with hirudin, fluorescence values were not significantly different from control values, suggesting that the primary mode of inhibition of shear aggregation by hirudin is not due to a decreased availability of activated GPIIb-IIIa* receptors. However, at the higher thrombin concentration tested, 0.2 and 1.0 U/ml, FITC-PAC-1 binding decreased in the presence of hirudin by 23 % (n=2) and by 37 $\pm$ 1 % (n= 3) ($p < 0.01$) respectively.

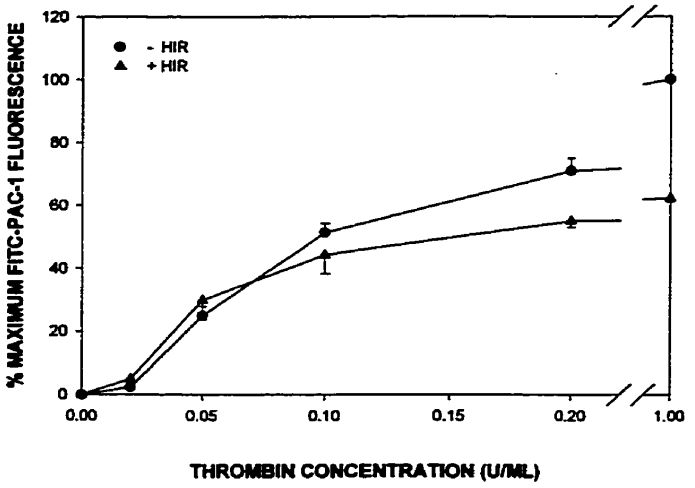

Fig.4-6. Expression of activated GPIIb-IIIa receptors in the presence or absence of thrombin antagonists. WP were incubated with mAb FITC-PAC-1 which recognizes the activated GPIIb-IIIa conformation, together with thrombin in absence of, or after treatment with antagonist, as in Fig. 4-4.

3.7. Effects of soluble fibrinogen on the aggregation of WP activated with 0.05 U/ml thrombin and treated with hirudin or PPACK: Since Fg was shown to bind to thrombin pre-activated WP treated with hirudin or PPACK, we considered that soluble Fg may be able to cross-bridge such platelets in shear flow, and thus increase α_G values. Incubation times with Fg were adjusted to give occupancies of activated GPIIb-IIIa receptors in the range of 20 - 75 %, previously shown to result in optimal platelet aggregation (31). Accordingly, the numbers of Fg molecules and numbers of activated GPIIb-IIIa receptors on sheared platelets were determined to be in the range of 4500-7100 and 11,000-14,000 respectively, yielding percent occupancies of 49 ± 5 % (n = 4). Surprisingly, the rate and extent of aggregation at G = 1000 s^{-1}, of antagonist treated platelets in the presence of 0.2 μM FITC-Fg, were not significantly different from that in the absence of soluble ligand (Fig. 4-7a), the change in α_G being -1.0 ± 11%, (n = 4) for hirudin and 5 ± 3% (n = 3), for PPACK. Similar results

were obtained at G = 300 s^{-1} (Fig. 4-7b), despite the important role expected for Fg at lower shear rates (37).

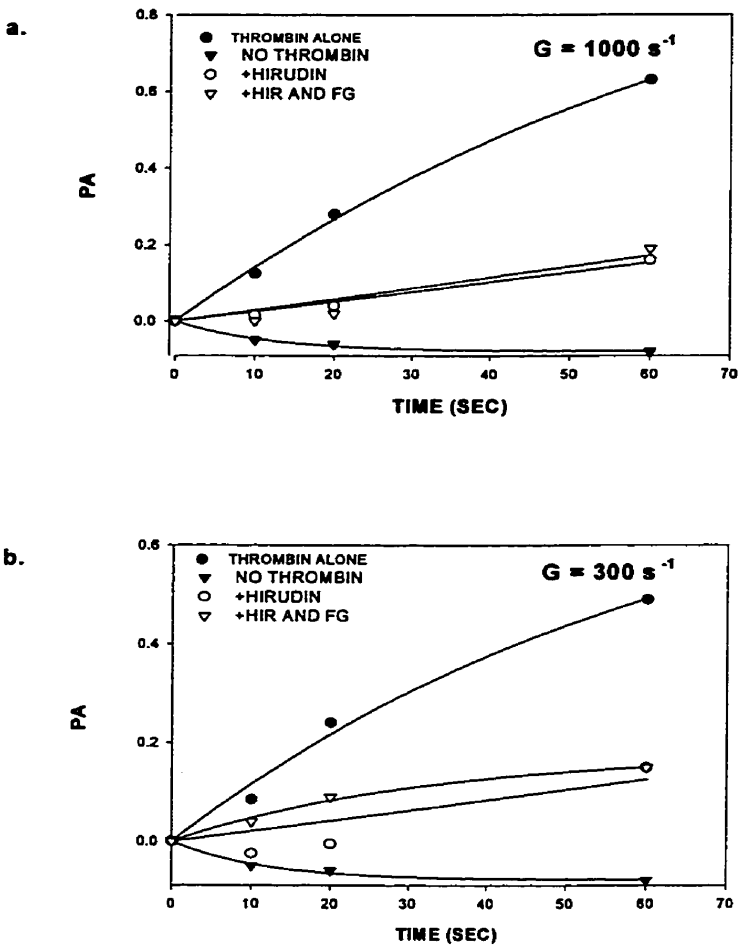

Fig. 4-7. Effect of soluble Fg on the kinetics of aggregation of WP pre-activated with 0.05 U/ml thrombin and treated with hirudin after 10 minutes. WP (4 x $10^4/\mu l$) activation with thrombin and antagonist addition were as in fig. 1. To examine the effect of soluble Fg on the shear-associated aggregation, platelets were incubated for a further 5 minutes after hirudin addition, with 0.2μM Fg prior to shearing at *a.* G = 1000 or *b.* = 300 s^{-1}. Results shown are for a representative donor.

4. DISCUSSION

In studies by Holmsen et al and Greco et al (10,11) on requirements for thrombin occupancy of platelet receptors for secretion and aggregation with 0.025 - 0.12 U/ml thrombin, it was concluded that secretion was the key event regulating the occurrence of platelet aggregation in flow; hence, once the lag phase corresponding to the onset of secretion had passed (45 - 60 s), no further requirement for thrombin receptor occupancy for platelet aggregation was postulated. We have confirmed the complete absence of aggregation of thrombin-activated washed platelets in suspension, subjected to stir or laminar shear flow, in the presence of an 8-fold excess of hirudin, or 2 μM PPACK, or when these antagonists were added after thrombin, but within the time required for secretion. However, differences in methodology, in particular timing, have allowed us to uncouple requirements for thrombin receptor occupancy for surface expression of secreted proteins and upregulation of GPIIb-IIIa receptor ligand recognition, from those for initiation of aggregation. Additionally, we have shown that platelets which have been treated with hirudin or PPACK after 10 minutes of thrombin activation ("pre-activated" platelets), differ in their requirement for thrombin occupancy of its receptors dependant on the thrombin concentration used to activate the platelets. Thus, for WP pre-activated with 0.05 U/ml thrombin and treated with antagonist, occupancy of thrombin receptor(s) by catalytically active thrombin: 1) was required for optimal capture efficiencies, for maintenance of surface expression of vWF and TSP, and for activated GPIIb-IIIa receptor recognition of soluble vWF; and 2) was independent of surface expression of α-granule membrane P-selectin or of availability of activated GPIIb-IIIa

receptors for Fg binding. When WP were similarly treated with antagonist, but pre-activated with 0.2 U/ml thrombin, all parameters tested above were independent of thrombin receptor occupancy.

4.1. Comparison of high versus low thrombin concentrations

We have consistently found that platelets activated with high thrombin concentrations (0.2 - 1 U/ml) did not show a dependence on occupancy of thrombin receptor(s) for continued optimal aggregation, surface vWF or TSP expression, or in binding characteristics of the activated GPIIb-IIIa receptor. This is in contrast to the dependence seen for low thrombin concentrations (0.05 U/ml). At low thrombin concentrations, primarily the high-affinity receptor, GPIb, would be occupied , while at 0.2 - 1 U/ml thrombin, both the GPIb receptor and the moderate affinity thrombin 7-transmembrane receptor (PAR-1) would participate in mediating responses by thrombin (12), with irreversible cleavage of the amino-terminal domain of PAR-1 ensuring hirudin-resistant signalling through this receptor. In addition, the extent and nature of secretion appear distinct for the low and high thrombin concentrations (19,26), with increased % P^* for platelet surface expression, and perhaps stabilization, of secreted ligands. Detailed studies of secretion and surface binding of ligands with platelet activation need to be made to clarify the above.

4.2. Role for surface-expressed vWF and TSP in aggregation

In a previous study we demonstrated an important role for surface-expressed vWF (SE-vWF) as the principal cross-bridging agent of thrombin-activated platelets in shear flow

(21). This dependence on SE-vWF is also supported by our current data, since hirudin and PPACK reduced capture efficiencies only at the lower thrombin concentrations, where decreases in α_G were correlated with decreases in the % P^* maximally expressing surface-bound vWF. If all P^* are recruited into aggregates, and no P^0 (resting platelets), then a 40% reduction in % P^* would yield a 40% reduction in initial rates of aggregation, as reported for Fg-mediated aggregation of ADP-activated platelets (31,38). However, the percent decrease in α_G for sheared platelets activated with 0.05 U/ml thrombin, is greater for individual donors than the % decrease observed in % P^* for SE-vWF, likely reflecting contributions due to reductions in numbers of surface-bound vWF and TSP (Fl^*), % P^* for SE-TSP, or other factors. The specific role of TSP was not investigated further, but several studies have suggested that TSP may be important for stabilization of aggregate formation (39) and for amplification of the secretion response (40).

4.3. Effect of hirudin and PPACK on soluble Fg and vWF binding

Binding of soluble vWF was almost completely abolished by the addition of hirudin or PPACK 10 minutes after platelet activation with low concentrations of thrombin, whereas the normal occupancy of receptors by soluble Fg binding appears to correspond to the availability of activated GPIIb-IIIa, shown via PAC-1, an anti-GPIIb-IIIa activation-dependent antibody. Since the numbers of GPIIb-IIIa* receptors were not reduced by neutralization with thrombin antagonists, it would appear that GPIIb-IIIa conformation may be compromised, with perhaps more stringent requirements for the vWF- GPIIb-IIIa interaction than for Fg. Any direct effects of hirudin or PPACK, two very distinct molecules,

on both vWF (soluble and SE- vWF) and SE-TSP, are unlikely and would be expected to be even more pronounced at 0.2 U/ml thrombin, where larger concentrations of hirudin and PPACK were used. Moreover, we have demonstrated the lack of direct interference with vWF-GPIb or vWF- GPIIb-IIIa interactions by hirudin, and vWF-GPIIb-IIIa interactions by PPACK, using ristocetin and model latex particles with surface-bound activated GPIIb-IIIa.

Although many earlier studies indicate that there are three conformational states of GPIIb-IIIa: unactivated, activated unoccupied, and activated but ligand occupied, there are several lines of evidence suggesting that further fine tuning of conformations presented may exist, depending on activating conditions and ligands bound. The recently described anti GPIIb-IIIa mAb, PMA-5, can selectively inhibit Fg-mediated aggregation of ADP-activated platelets (and hence GPIIb-IIIa), but not the aggregation of RGD-activated fixed platelets (41), while mAb AP-6, recognizing a ligand-induced binding site on residues 203-228 on GPIIIa (LIBS) can distinguish between RGD or γ 400-411 peptide -occupied GPIIb-IIIa and the receptor occupied by the intact ligand, Fg (42).

It has been shown that the γ 400-411 site on Fg is exclusively required for binding to activated GPIIb-IIIa (43) and for cross-bridging of platelets (44), and that this epitope has been chemically cross-linked to a domain consisting of residues 296-306 on GPIIb (45), whereas the RGD domain on vWF is expected to bind to region 109-171 on GPIIIa (46). Thus, perturbations of the final steps of the normal signal transduction pathway for low-dose thrombin GPIb-mediated activation, controlling ligand affinities and duration of activated conformational states (47,48), may result in altered stability or activated GPIIb-IIIa conformation in the absence of thrombin occupancy, with differentially exposed epitopes for

the binding of PAC-1, Fg and vWF.

4.4. Effect of soluble Fg on platelet aggregation

The γ 400-411 recognition domain on GPIIb although likely to be available as suggested by Fg binding, may nevertheless have reduced accessibility in thrombin-antagonist treated platelets, as suggested by the inability of soluble Fg to improve α_G values at intermediate shear rates of G = 1000 s^{-1}, and even at G = 300 s^{-1}, where Fg is at its peak efficiency (31). The apparent inability of soluble Fg to cross-bridge platelets following binding to platelets has been described previously for effects of a combination of intact monoclonal antibodies against GPIIb and against GPIIIa (49), and for platelets activated with ADP at 4^0 C (50), but ascribed to post-Fg binding events. Similar effects of hirudin and PPACK on post-ligand binding events remain a possible explanation for Fg behaviour, although this would not explain the decrease observed in the binding of soluble vWF. The internalization of GPIIb-IIIa receptors with bound Fg reported by Wencel-Drake et al.(51) as a mechanism to explain reduced aggregation of platelets in stirred suspensions, is an unlikely explanation for our experiments, since after five minutes of incubation with Fg, Wencel-Drake et al. indicated that the extent of decrease in surface-Fg at this time point resulted in only a 20 % inhibition of aggregation. Thus, sufficient Fg should remain bound to platelets to participate in the mediation of aggregation, and to increase α_G values in our system.

4.5. Concluding remarks

We have shown an uncoupling of the requirement for thrombin occupancy for the secretion versus aggregation response in platelets treated with hirudin or PPACK after an extended thrombin activation period. Inhibition of aggregation at low thrombin activating concentrations was correlated with a decreased percentage of activated platelets with surface expression of vWF and TSP, reduced soluble vWF binding and abnormal Fg-mediated aggregation. Thus, in clinical trials with patients receiving hirudin or PPACK-based thrombin antagonists, where some low levels of thrombin generation may occur, initial rates of thrombin-induced platelet aggregation are likely to be reduced at the low thrombin concentrations present initially, and may contribute to the bleeding complications observed in some patients.

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6. REFERENCES

1. Naski MC, Fenton JW II, Maraganore JM, Olson ST, Shafer JA. The COOH-terminal domain of hirudin. An exosite-directed competitive inhibitor of the action of alpha thrombin on fibrinogen. J Biol Chem 1990; 265:13484-89.

2. Rydel TJ, Tulinsky A, Bode W, Huber R, Roitsch C, Fenton JW,II. Refined structure of the hirudin-thrombin complex. J Mol Biol 1991; 221: 583-601.

3. Holmsen H, Dangelmaier CA, Rongved S. Tight coupling of thrombin-induced acid hydrolase secretion and phosphatidate synthesis to receptor occupancy in human platelets. Biochem J 1984; 222:157-67.

4. Jandrot-Perrus M, Didry D, Guillin MC, Nurden A. Cross-linking of α– and γ–thrombin to distinct binding sites on human platelets. Eur J Biochem 1988; 174: 359-67.

5. Yamamoto N, Greco NJ, Barnard MR, Tanque K, Yamazaki H, Jamieson GA, Michelson AD: Glycoprotein Ib (GPIb)-dependent and GPIb-independent pathways of thrombin-induced platelet activation. Blood 1991; 77: 1740-8.

6. Topol EJ, Fuster V, Harrington RA, Califf RM, Klieman NS, Kereiakes DJ, Cohen M, Chapekis A, Gold HK, Tannenbaum MA, Rao AK, Debowey D, Schwartz D, Henis M, Chesebro JH. Recombinant hirudin for unstable angina pectoris. A multicentre randomised angiographic trial. Circulation 1994; 89: 1557-66.

7. Eriksson BI, Ekman S, Kälebo P, Zachrisson B, Bach D, Close P. Prevention of deep-vein thrombosis after total hip replacement: direct thrombin inhibition with recombinant hirudin CGP 39393. Lancet 1996; 347: 635-9.

8. van den Bos AA, Deckers JW, Heyndrickx GR, Laarman G-J, Suryapranata H, Zijlstra

F, Close P, Rijnierse JJMM, Bűller HR, Serruys PW. Safety and efficacy of recombinant hirudin (CGP 39393) versus heparin in patients with unstable angina undergoing coronary angioplasty. Circulation 1993; 88: 2058-66.

9. Global Use of Strategies to Open Occluded Arteries (GUSTO) IIa Investigators. Randomized trial of intravenous heparin versus recombinant hirudin for acute coronary syndromes. Circulation 1994; 90: 1631-7.

10. Holmsen H, Dangelmaier CA, Holmsen HK. Thrombin-induced platelet responses differ in requirement for receptor occupancy; evidence for tight coupling of occupancy and compartmentalized phosphatidic acid formation: J Biol Chem 1981; 256: 9393-96.

11. Greco, NJ, Tenner Jr. TE, Tandon NN, Jamieson GA. PPACK-Thrombin inhibits thrombin-induced platelet aggregation and cytoplasmic acidification but does not inhibit platelet shape change. Blood 1990; 75:1983-90.

12. Greco NJ, Jones GD, Tandon NN, Kornhauser R, Jackson B, Jamieson GA. Differentiation of the two forms of GPIb functioning as receptors for alpha-thrombin and von Willebrand factor: Ca^{2+} responses of protease-treated human platelets activated with alpha thrombin and the tethered ligand peptide. Biochem 1996; 35: 915-21.

13. Plow EF, Pierschbacher MD, Ruoslahti E, Marguerie GA, Ginsberg MH. The effect of Arg-Gly-Asp-containing peptides on fibrinogen and von Willebrand factor binding to platelets. Proc Nat Acad Sci USA 1985; 82: 8057-61.

14. Kettner C, Shaw E. D-Phe-Pro-ArgCH_2CL- A selective affinity label for thrombin. Thromb Res 1979; 14: 969-73.

15. Schor K, Darius H, Matzky R, Ohlendort R. The antiplatelet and cardiovascular actions

of a new carbaxylic derivative (ZK 36374)- equipotent to PGI_2 in vitro. Arch Pharm 1981; 316: 252-55.

16. Coller BS, Peerschke EI, Scudder LE, Sullivan CA. Studies with a murine monoclonal antibody that abolishes ristocetin-induced binding of von Willebrand factor to platelets; additional evidence in support of GPIb as a platelet receptor for von Willebrand factor. Blood 1983; 61: 99-110.

17. Shattil SJ, Cunningham M, Hoaxie JA. Detection of activation platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. Blood 1987; 70: 307-15.

18. Goto S, Salomon DR, Ikeda Y, Ruggeri ZM. Characterization of the unique mechanism mediating the shear-dependent binding of soluble von Willebrand factor to platelets. J Biol Chem 1995; 270:23352-61.

19. Legrand C, Dubernard V, Kieffer N, Nurden AT. Use of a monoclonal antibody to measure the surface expression of thrombospondin following platelet activation. Eur J Biochem1988; 171: 393-9.

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

21. Kasirer-Friede A, Frojmovic MM. Ristocetin and thrombin-induced platelet aggregation at physiological shear rates: Differential roles for GPIb and GPIIb-IIIa receptor. Thromb Haemost, 1998; 80: 428-36.

22. P. Ramsamooj, G.J. Doellgast, R.R. Hantgan, Inhibition of fibrin(ogen) binding to stimulated platelets by a monoclonal antibody specific for a conformational determinant of

GPIIIa. Thromb Res 1990; 58 : 577-92.

23. Goldsmith HL, Frojmovic MM, Braovac S, McIntosh F, Wong,T. Adenosine diphosphate-induced aggregation of human platelets in flow through tubes: III. Shear and extrinsic fibrinogen-dependent effects. Thromb Haemost 1994; 71: 78-90.

24. Tang SS, Frojmovic MM. The effects of pCO_2 and pH on platelet shape change and aggregation for human and rabbit platelet-rich plasma. Thromb Res 1977; 10: 135-45.

25. George JN, Torres MM. Thrombin decreases von Willebrand factor binding toplatelet Glycoprotein Ib. Blood 1988; 71: 1253-59.

26. Legrand C, Dubernard V, Nurden AT. Studies on the mechanism of expression of secreted fibrinogen on the surface of activated platelets. Blood 1989; 73: 1226-34.

27. Huang EM, Detweiler TC. Thrombin-induced phosphoinositide hydrolysis in platelets. Biochem J 1987; 242: 11-18.

28. Prasa D, Svendsen L, Stürzebecher J. The ability of thrombin inhibitors to reduce the thrombin activity generated in plasma on extrinsic and intrinsic activation. Thomb Haemost 1997; 77: 498-503.

29. Jackman MP, Parry MA, Hofsteenge J, Stone SR. Intrinsic fluorescence changes and rapid kinetics of the reaction of thrombin with hirudin. J Biol Chem1992; 267:15375-83.

30. Frojmovic MM, Mooney RF, Wong T. Dynamics of platelet glycoprotein IIb-IIIa receptor expression and fibrinogen binding. I. Quantal activation of platelet subpopulations varies with adenosine diphosphate concentration. Biophys J 1994; 67: 2060-8.

31. Xia A, Frojmovic MM. Aggregation efficiency of activated normal or fixed platelets in a simple shear field: Effect of shear and fibrinogen occupancy. Biophys J 1994; 66: 2190-

2201.

32. Xia Z, Wong T, Liu Q, Kasirer-Friede A, Brown E, Frojmovic MM. Optimally functional fluorescein isothiocyanate-labelled fibrinogen for quantitative studies of binding to activated platelets and platelet aggregation. Br J Haematol 1996; 93: 204-14.

33. Van de Ven, TGM. Two colloidal particles subjected to an external field. In: Colloidal Hydrodynamics. Academic Press, London, pp. 384-91, 1989.

34. 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.

35. Born GVR, Hume M. Effects of the numbers and sizes of platelet aggregates on the optical density of plasma. Nature 1967; 215: 1027-9.

36. Suzuki H, Kinlough-Rathbone RL, Packham MA, Tanoue K, Yamazaki H, Mustard F. Immunocytochemical localization of fibrinogen during thrombin-induced aggregation of washed human platelets. Blood 1988; 71: 1310-20.

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

38. Frojmovic MM, Mooney RF, Wong T. Dynamics of platelet glycoprotein IIb-IIIa receptor expression and fibrinogen binding. II. Quantal activation parallels platelet capture in stir-associated microaggregation. Biophys J 1994; 67: 2069-75.

39. Leung LLK. Role of thrombospondin in platelet aggregation. J Clin Invest 1984; 74: 1764-72.

40. Rabhi-Sabile S, Thibert V, Legrand C. Thrombospondin peptides inhibit the secretion-dependent phase of platelet aggregation. Blood Coag Fibrinolys 1996; 7: 237-40.

41. Nakatani S, Hato T, Minamoto Y, Fujita S: Differential inhibition of fibrinogen binding to agonist-and RGD peptide-activated states of GPIIb-IIIa by an anti-GPIIIa monoclonal antibody. Thromb Haemost 1996; 76: 1030-7.

42. Nurden P, Humbert M, Piotrowicz RS, Bihour C, Poujol C, Nurden AT, Kunicki TJ. Distribution of ligand-occupied $\alpha_{IIb}B_3$ in resting and activated human platelets determined by expression of a novel class of ligand-induced binding site recognized by monoclonal antibody AP-6. Blood 1996; 88: 887-99.

43. Amrani DL, Newman PJ, Meh D, Mosesson MW. The role of fibrinogen Aα chains in ADP-induced platelet aggregation in the presence of fibrinogen molecules containing γ' chains. Blood 1988; 72: 919-24.

44. Liu Q, Matsueda G, Brown E, Frojmovic MM. The AGDV residues on the gamma chain carboxyl terminus of the platelet-bound fibrinogen are needed for platelet aggregation. Biochimica Biophysica Acta 1997; 1343: 316-26.

45. D'Souza SE, Ginsburg MH, Matsueda GR, Plow EF. A discrete sequence in a platelet integrin is involved in ligand recognition. Nature 1991; 350: 66-8.

46. 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.

47. Giesberts AN, van Willigen G, Lapetina EG, Akkerman JN. Regulation of platelet glycoprotein IIb/IIIa (integrin $\alpha_{IIb}\beta_3$) function via the thrombin receptor. Biochem J 309: 613-620, 1995

48. van Willigen G, Hers I, Gorter G, Akkerman JWN: Exposure of ligand-binding sites on integrin α_{IIB} /B_3 by phosphorylation of the B_3 subunit. Biochem J 1996; 314: 769-79.

49. Newman PJ, McEver RP, Doers MP, Kunicki TJ. Synergistic action of two murine monoclonal antibodies that inhibit ADP-induced platelet aggregation without blocking fibrinogen binding. Blood 1987; 69: 668-76.

50. Peerschke EI, Zucker MB. Fibrinogen receptor exposure and aggregation of human blood platelets produced by ADP and chilling. Blood 1981; 57: 663-9.

51. Wencel-Drake JD, Boudignon-Proudhon C, Dieter MG, Criss AB, Parise LV. Internalization of bound fibrinogen modulates platelet aggregation. Blood 1996; 87: 602-12

Introduction to Chapter 5

Thrombospondin and fibrinogen have been implicated in thrombin-induced macro-aggregation of platelets in stirred suspensions. In this study, we evaluate the contribution of these adhesive ligands as well as vWF, to initial rates of aggregation of platelets in uniform laminar shear flow, at physiologic shear rates between $G = 300$ and $2000\ s^{-1}$. Differences in receptor-ligand requirements resulting from different exposure times to thrombin prior to placement in the shear field, was also determined.

Chapter 5

Complementary Roles for Fibrinogen, Thrombospondin and von Willebrand Factor in Mediating Shear-Dependent Aggregation of Platelets Stimulated at Threshold Thrombin Concentrations

Abstract

Although antibodies against several adhesive ligands have been shown to inhibit the aggregation of stirred washed platelets (WP) activated with low concentrations of thrombin (0.05 U/ml), the specific role of individual ligands has not been well established. We have investigated the contributions of von Willebrand factor (vWF), fibrinogen (Fg) and thrombospondin (TSP), to the efficiency of platelet capture at various physiologic shear rates (G = 300 (≈venous), 1000 or 2000 s^{-1} (≈microcirculation)) and pre-incubation conditions (30 s (τ_0) or 10 minutes (τ_{10})) with threshold thrombin concentrations. Fluorescently-labelled reporting antibodies, detected by flow cytometry, confirmed the expression of vWF, Fg, and TSP, on activated platelet surfaces. vWF was essential for mediating platelet aggregation at all shear rate and incubation conditions tested. Antibodies against TSP inhibited initial rates of aggregation in a shear-rate independent manner by 35-65 %. For platelets pre-incubated with thrombin (τ_{10}), Fg did not directly cross-bridge platelets in flow, although a secondary, supportive role for Fg is postulated, potentially in its proteolysed form, fibrin. At τ_0, vWF, TSP and Fg all appeared to mediate aggregation, with Fg now directly cross-bridging platelets via the γ-carboxyl terminus. Thus, optimum shear-associated aggregation of WP activated with low thrombin concentrations requires all three adhesive ligands, with diverse role(s) postulated for each, depending on shear and activating conditions.

1. INTRODUCTION

In hemostasis, vascular injury results in exposure of subendothelium and the rapid adhesion of flowing platelets to matrix proteins, as demonstrated for von Willebrand factor (vWF) and collagen (1), with subsequent activation of platelets. Platelet activation may be associated with release of α-granule proteins and platelet surface expression of adhesive ligands, including fibrinogen(Fg), vWF and thrombospondin (TSP) (2). Further recruitment of platelets for aggregate growth may be mediated by thrombin or released ADP (3,4). VWF and Fg have also been implicated in both venous and arterial thrombosis (5,6), while TSP levels are elevated in the thickened intima in human vascular disease (7).

Antagonists against adhesive ligands or glycoprotein receptors can inhibit the macro-aggregation of washed platelets activated with thrombin (8-10). However, the decrease in aggregation may be due to direct inhibition of cross-bridging sites on ligand or receptor, or by inhibition of processes required for optimal platelet surface presentation of ligands following activation. Platelet binding and participation in aggregation of a given ligand may be mediated by homotypic platelet receptors (eg. GPIIb-IIIa*-Fg-GPIIb-IIIa*), or by heterotypic receptors (eg.GPIb-vWF-GPIIb-IIIa*) (11-14), as summarized in Fig.5-1. Direct associations between adhesive ligands have also been proposed (Fig.5-1), which include TSP with vWF (15), Fg (17) or fibrin (Fn) (11), and vWF with Fn (16). These ligands may also serve to amplify the secretion process, as reported for TSP in thrombin-mediated aggregation (18).

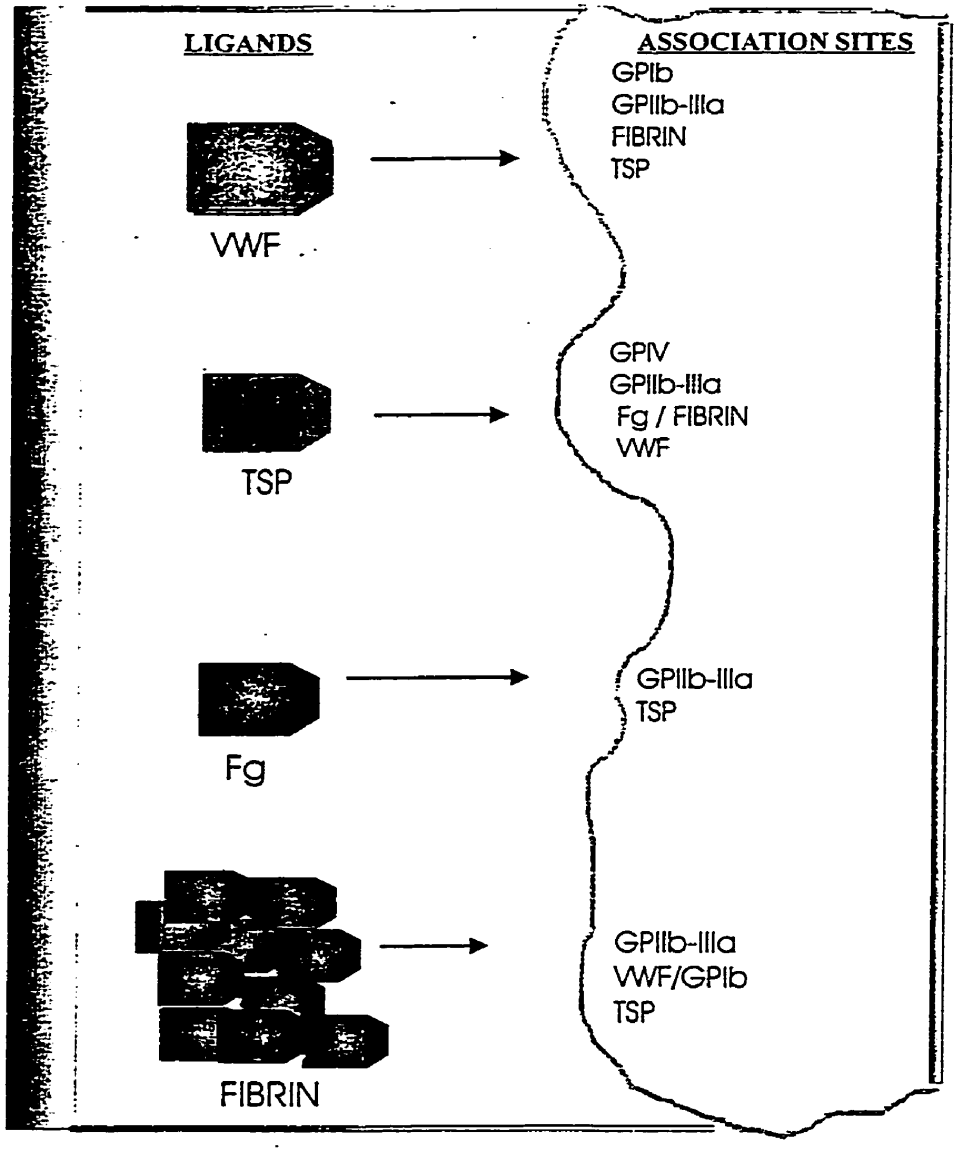


Figure 5-1. Potential association sites between soluble or surface-bound adhesive ligands von Willebrand factor (vWF), thrombospondin (TSP), fibrinogen (Fg), and fibrin and glycoprotein receptors, for platelet surface presentation or cross-bridging.

We have previously demonstrated an important role for vWF in optimizing initial rates of aggregation of washed platelets, activated with low concentrations of thrombin or ADP, and sheared in laminar flow at shear rates G = 300 or 1000 s^{-1} (19-21). The role of the two other adhesive ligands, TSP and Fg, has not been previously investigated under similar conditions. We have used a panel of antibodies against Fg, TSP, and vWF to determine their role in platelet aggregation under conditions of uniform laminar shear flow. Platelets were sheared at G = 300 - 2000 s^{-1}, at threshold thrombin activating concentrations, and with varying incubation times.

2. METHODS

2.1. Reagents:

Phorbol myristyl-acetate (PMA), ADP, and α-thrombin were purchased from Sigma Corp. USA; 8% glutaraldehyde, electron microscopy grade, from Polysciences Inc.; fluorescein-isothiocyanate (FITC; Isomer I) from Boehringer Diagnostics, La Jolla, CA).

2.2 Reporting and inhibitory antibodies

Monoclonal antibodies (mAbs) used were (also listed in Table 1 for easy reference): mAb S.12 (IgG1), against platelet membrane P-selectin (22), a gift from Dr. McEver, Oklahoma Medical Research Foundation, Oklahoma City, OK; mAb 2.2.9., (IgG1), recognizes a non-functional epitope between vWF residues 1366-2050 (23), generously provided by Dr. Z. Ruggeri, Scripps Institute, La Jolla, CA; mAb 4A5, (IgG1 $_{\kappa}$), recognizing γ 402-411 on

native Fg or fibrin, inhibits soluble fibrinogen-mediated platelet aggregation and adhesion (24), generously provided by Dr. G. Matsueda, Princeton, N.J.. MAb MAII, (IgG1) against the heparin binding domain (HBD) on TSP (25), inhibits binding to Fg, and ADP or thrombin-mediated platelet aggregation (9) and mAb MAI, (IgG1) against the TSP type 3 repeat region (25), minimally inhibiting thrombin-mediated aggregation (9), were a generous gift of Dr. J. Lawler, Harvard Medical School, Boston MA; mAb 5G11 (IgG_{2a}) against a non-functional epitope on TSP (26); and polyclonal affinity-purified $F(ab')_2$ fragments against TSP, generously contributed by Diagnostica Stago, France, previously shown to inhibit thrombin-induced aggregation (27). $F(ab')_2$ fragments from a nonimmune rabbit were prepared as control. Polyclonal $F(ab')_2$ fragments against Fg, shown by us to inhibit soluble-Fg mediated aggregation of ADP-activated platelet rich plasma, and non-immune $F(ab')_2$ fragments to IgG were purchased from ICN Chemicals in Montreal, Canada. A polyclonal IgG Ab against vWF, inhibiting thrombin, or ristocetin and soluble vWF-mediated aggregation of washed platelets (20), and control non-immune IgG were from Cedarlane Laboratories Ltd. in Montreal, Canada; The mAb T2G1, (IgG1 κ) against Bß 15-42 on fibrin II (28), was a generous gift from Dr. B. Kudryk, New York Blood Center, New York, NY.

2.3. Preparation of washed platelets and labelling of monoclonal antibodies: Washed platelets (WP) were prepared by a one-step wash procedure from human citrated whole blood as previously described (29). Antibodies were labelled with fluorescein-isothiocyanate (FITC), and fluorescein to protein ratios (F:P) were calculated (30).

2.4. Binding experiments: In a previous study, we had shown a threshold thrombin activating concentration for both the onset of platelet aggregation and for the fraction of platelets expressing increased levels of vWF, occurring at identical EC_{50}'s (20). We therefore determined the thrombin threshold concentration (reflecting very low thrombin concentrations available early in hemostasis) for each donor, using reporting antibody FITC-2.2.9 to examine the surface expression of vWF (SE-vWF). Five μl of WP, at 4 x 10^4 /μl in a modified Tyrode buffer (BAT:136 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 1.0 mM $MgCl_2$, 5.6 mM glucose, 0.35% BSA) with 1mM Ca^{2+}, were added to FACS tubes, activated with thrombin at concentrations between 0.02 and 0.07 U/ml in increments of 0.01 - 0.02 U/ml, and incubated for 30 minutes with 400 nM FITC-2.2.9.. The reaction was arrested using a 10-fold dilution with BAT buffer, and the average fluorescence per platelet particle read immediately on a flow cytometer, FACScan (Becton Dickinson, Mississauga, Ont), as previously described (20,21). Fluorescence for the total cell population is reported as Fl_{tot}. In most cases, platelet populations showed a bimodal fluorescence distribution due to resting (P^0) and activated platelets (P^*), where fluorescence values were increased over background values in the absence of activator (Fl^*). The threshold thrombin concentration was defined as that at which the % P^* for FITC-2.2.9 increased by at least double, to a minimum of at least 40 % over background values. This concentration was used for further binding and aggregation studies.

2.4.1. Surface expression of TSP, P-selectin and Fg: In order to determine the surface expression of α-granule-proteins other than vWF on platelets activated with thrombin, WP

at $10^4/\mu l$ in BAT buffer with 1 mM Ca^{2+}, were activated in the presence of reporting antibodies: FITC-5G11 (333 nM) or FITC-S.12 (273 nM), used to detect surface-expressed TSP (SE-TSP) and P-selectin respectively, and incubated for 30 minutes. Fluorescence values were determined as described above. SE-Fg was assessed using FITC-4A5 (110 nM), added 10 minutes after activation, to permit normal Fg binding to platelets.

2.4.2. Effect of inhibitory antibodies used for functional tests, on the surface expression of adhesive ligands: Binding with reporting antibodies was performed as above, except that inhibitory antibodies, were added immediately prior to thrombin activation at the following concentrations, previously shown to inhibit platelet aggregation in stirred suspensions, or determined in our laboratory: 1.3 μM polyclonal $F(ab')_2$ anti-Fg (29) or non-immune IgG , 3.9 μM polyclonal anti-vWF or control IgG, 67 nM polyclonal $F(ab')_2$ anti-TSP (27) or control IgG, 300 nM MAII or MAI (9). The binding of reporting antibody to WP in the presence of inhibitor was expressed as the percentage inhibition of control fluorescence obtained in the absence of antagonist.

2.4.3. Detection of fibrin formation: A fluorescently-labelled mAb against the Bß 15-42 residues on fibrin II (FITC-T2G1) was incubated with WP ($10^4/\mu l$) , BAT, 1 mM Ca^{2+}, and activated with thrombin, or with 0.2 μM PMA . Platelet suspensions were incubated for 10 or for 30 minutes, at which time the reaction was arrested by dilution, and the fluorescence values read on the FACSCAN.

2.5. Aggregation in shear:

Aggregation of WP under uniform laminar shear was tested in a coaxial cylinder microcouette (0.3 mm gap), as previously described (21,31). Sheared aliquots were immediately fixed in 10 vol of 0.2 % glutaraldehyde, then diluted with isotonic saline for particle counting using the FACScan. Platelet aggregation (PA) and capture efficiency α_G were respectively determined from the decrease in particle number with time, and from the ratio of the rate of two-body collisions resulting in aggregate formation to the total rate of two-body collisions, as previously described by Xia and Frojmovic (31).

2.5.1. Thrombin-induced shear-associated aggregation of WP: Platelet suspensions (4 x $10^4/\mu$l) were pre-mixed in Eppendorf tubes with BAT buffer containing 1mM Ca^{2+}, and thrombin, and immediately transferred to the microcouette for shearing (τ_0). At τ_0, inhibitory antibodies against TSP, vWF and Fg, were added to platelets 0-2 minutes prior to thrombin, at concentrations used in binding experiments. Alternatively, platelets were pre-incubated with thrombin for 10 minutes (τ_{10}) prior to transfer to the microcouette. In these experiments, inhibitory antibodies were added either prior to or at the end of the incubation period. Results reported for percentage inhibition of aggregation (% I α_G) by vWF and TSP antibodies were corrected for inhibition obtained with non-immune control antibodies. The % I of α_G for TSP for two different antibodies were very similar at all conditions tested. As an example, at G = 300 s^{-1}, the % I of α_G were 47 ± 12 % (n = 4) and 48.5 % (n = 2) for MAII and for the polyclonal F(ab')$_2$ anti-TSP fragments respectively. Results were therefore pooled for these two antibodies.

2.6.Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM), with numbers in parentheses indicating the number of experiments performed. Tests for statistical significance were performed using the Student t-test.

3. RESULTS

3.1. Surface Expression of adhesive ligands:

The thrombin threshold concentration determined using mAb FITC-2.2.9. occurred at 0.04 ± .003 U/ml, with a range between 0.02 - 0.07 U/ml, and resulted in 35 - 60 % of platelets showing maximal binding (% P^*). This activating concentration, determined for each WP preparation, was used for further binding and functional studies. Henceforth, use of the term thrombin will indicate thrombin at threshold activating concentrations.

The specific binding of fluorescently-labelled reporting antibodies FITC-2.2.9, FITC-5G11 and FITC-4A5 (added 10 minutes after thrombin activation) against SE-vWF, SE-TSP, and SE-Fg respectively, increased on thrombin-activated platelets, demonstrating surface expression of all three adhesive ligands (Fig. 5-2). At these low thrombin concentrations, platelet suspensions contained subpopulations of activated platelets (% P^* ≥ 35 %), with the mean fluorescence for P^* (Fl^*) being approximately 95 % of the maximum Fl_{tot} for vWF and at least 50 % of the maximum Fl_{tot} for the other two ligands.

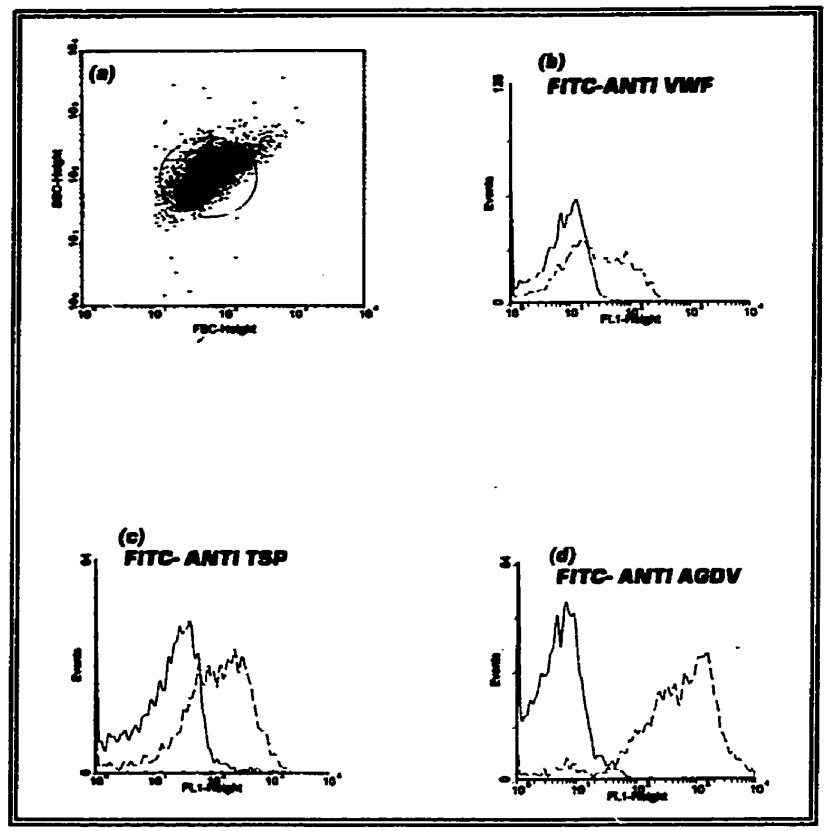


Figure 5-2. Surface expression of adhesive ligands vWF, TSP and Fg on washed platelets. WP (10^4/ μl) were incubated with or without threshold thrombin concentrations with reporting antibodies FITC-2.2.9, FITC-5G11 and FITC-4A5 against vWF (b.), TSP (c.) and anti AGDV on Fg (d.) respectively, and incubated for 30 minutes. Scatter profiles (a.) were gated to exclude platelet aggregates, and fluorescence histograms were determined for gated populations. Figures show the binding of reporting antibodies in the presence (dashed lines) and absence of agonist (solid lines), for a representative donor.

The surface expression of vWF, TSP, and Fg on thrombin-activated WP was determined in the presence and absence of functionally-inhibitory antibodies (Fig.5-3) to test inhibition specificity. A probe for the surface expression of P-selectin (FITC-S.12) was included to indicate α-granule secretion. Polyclonal $F(ab')_2$ fragments against Fg did not alter the surface expression of non Fg ligands. Two antibodies: mAb 4A5, against γ 402-411

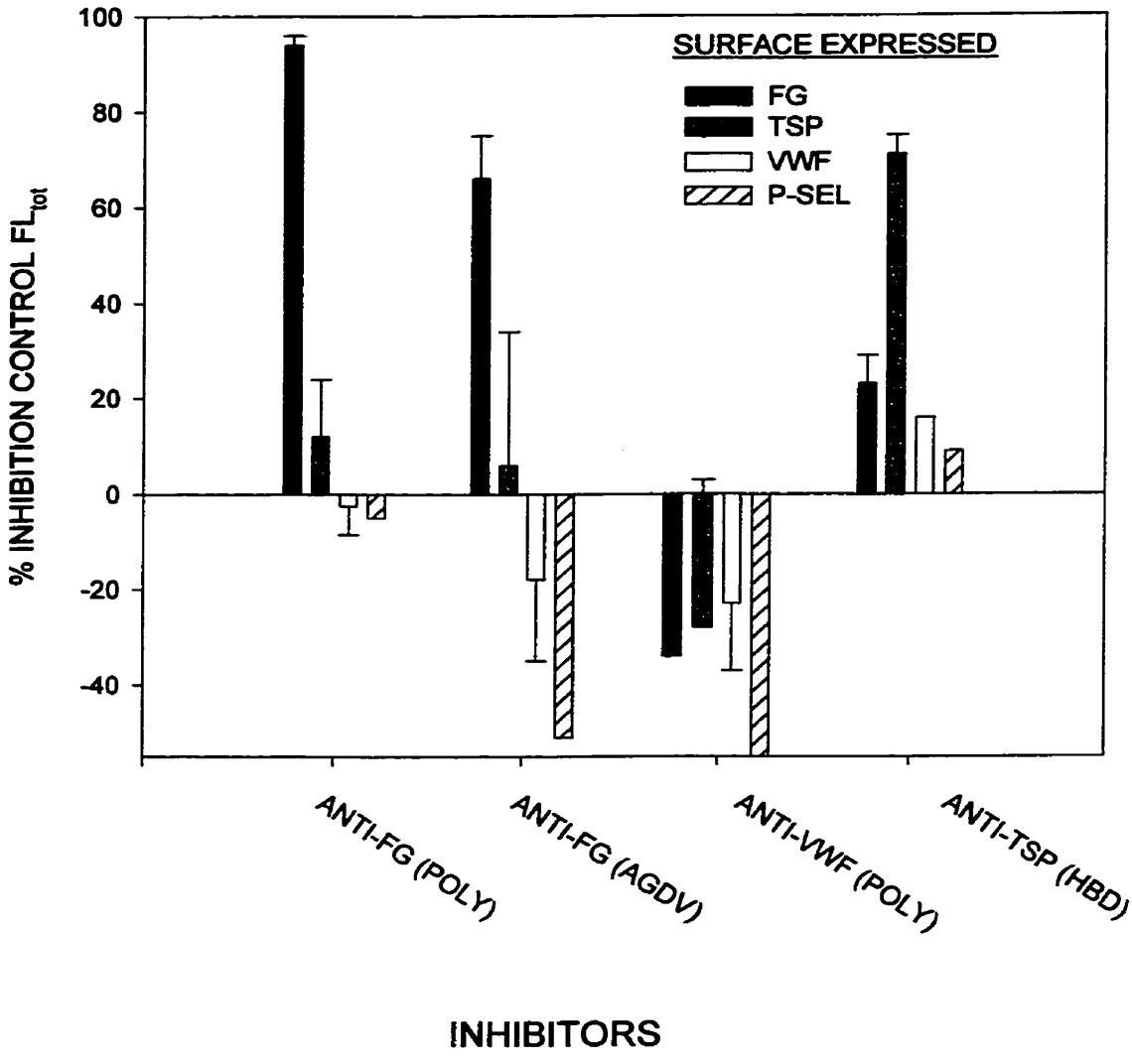


Figure 5- 3. Effect of functionally inhibitory antibodies on the surface expression of adhesive ligands. WP were incubated with thrombin either in the presence or absence of inhibitory antibodies against TSP (anti TSP-heparin binding domain), vWF (polyclonal) or Fg (anti AGDV on Fg or polyclonal F(ab')$_2$ fragments against Fg), as in Fig. 2. Total fluorescence (Fl$_{tot}$) was determined from fluorescence histograms. Results are presented as mean values for % inhibition of control (no inhibitor) fluorescence values ± SEM, for 3-6 donors for FITC- 2.2.9 and FITC-5G11 and at least 2 donors for FITC-S.12 and FITC-4A5, done in duplicate.

on Fg, and a polyclonal antibody against vWF, potentiated the surface expression of P-selectin by 1.2 to 3- fold. Although both Fg antibodies inhibited FITC-4A5 binding by 66 - 94 % (added at τ_{10}; Fig. 5-3), fluorescence values remained higher than with PMA activation (results not shown) which maximally drives platelet activation and secretion (32) but lacks proteolytic activity. A component of FITC-4A5 binding to thrombin-activated platelets may thus be attributable to fibrin. A mAb against the heparin-binding domain of TSP (MAII) altered the surface presentation of only TSP, with a significant decrease in Fl_{tot} of FITC-5G11 of 71 ● 4 % (n = 5; P<0 .01) (Fig. 5-3), similar to previously reported results using radiolabelled mAb 5G11 (26). There was no significant inhibition of the surface expression of any of the adhesive ligands by the isotype control, or of polyclonal $F(ab')_2$ fragments against TSP .

3.2. Aggregation in Shear:

The roles of vWF, TSP and Fg in mediating shear-associated platelet aggregation were tested under two different activating conditions. Platelets were activated and immediately transferred to the microcouette device for shearing (τ_0), or were pre-incubated for 10 minutes and then sheared (τ_{10}). For τ_{10}, inhibitors were added either prior to or after the pre-incubation period. Comparison of the inhibition of early rates of aggregation by these antibodies, when added before versus after a 10 minute pre-incubation with thrombin, should discriminate between primary events such as ligand secretion-amplifying functions or ligand stabilization, and secondary cross-bridging events, entailing direct participation in the capture of platelets in flow. After 10 minutes, we would not expect these ligands to play

further roles in the secretion process (21), and any inhibition of α_G may be attributed to participation in platelet aggregation by direct cross-bridging.

Washed platelet suspensions pre-activated at thrombin threshold concentrations, showed significant aggregation with % PA_{60s} increasing from 25 to 79 %, in agreement with previous results showing correlations between SE-vWF and % PA at the thrombin threshold concentration (20). Capture efficiencies (α_G) reflecting initial rates of aggregation (τ_{10}), were α_G = 0.2 ±0.03, 0.08 ± 0.01 and 0.06 ± 0.01 (n = 10) at G = 300, 1000 and 2000 s^{-1} respectively, with ≤15% lower α_G at τ_0.

3.2.1. Pre-activation of WP with thrombin, τ_{10} ; Role of vWF and TSP: A polyclonal anti-vWF antibody significantly inhibited α_G at all three shear rates tested (G = 300 - 2000 s^{-1}) (Table 5-1), with a significant difference in the extent of inhibition of α_G between the lowest and the 2 higher shear rates ($P< 0.05$). Antibodies against TSP also significantly inhibited α_G at all G, with no significant difference found for the % I α_G obtained at different G's. This is the first time that a role for TSP has been demonstrated in the shear-associated microaggregation of WP activated with thrombin.

Donor-matched data (n = 3) showed significantly less inhibition of α_G's by anti-TSP antibodies when added after 10 minutes pre-incubation with thrombin than when added prior to thrombin: 45 % less at G = 300 and 2000 s^{-1} ($P< 0.01$) and 60 % less at G = 1000 s^{-1} ($P< 0.01$)(data not shown). Thus, for TSP, we postulate both a role in platelet aggregation via cross-bridging, as reflected by the 35-50 % inhibition by TSP antibodies when added after a pre-incubation with thrombin, as well as an additional role reflecting the higher inhibition

when added prior to agonist. The polyclonal antibody against vWF strongly inhibited α_G when added after a 10 minute pre-activation with thrombin (post thrombin in Table 5-1), with inhibition of α_G almost twice as high as that by anti-TSP antibodies, at G = 2000 s^{-1}. Thus, as in previous studies (20,21), vWF appears to have an important, direct role in thrombin-mediated platelet aggregation at all G tested.

Table 5-1: Effect of inhibitory antibodies on the shear-associated aggregation of washed platelets pre-activated with threshold thrombin concentrations for 10 minutes

INHIBITORY ANTIBODY[a]	G (s^{-1})	% INHIBITION of α_G [b]	
		FOR ANTIBODY ADDED: PRE THROMBIN	POST THROMBIN
ANTI-VWF	300	74 ± 10 [c](3) §	63 ± 1 (3) //
	1000	95 ± 3 (3) §	67 ± 10 (3) //
	2000	92 ± 2 (6)	75 ± 4 (3) //
ANTI - TSP	300	48 ± 8 (6) //	50 ± 8 (4) //
	1000	65 ± 8 (9) //	37 ± 8 (5) //
	2000	58 ± 14 (4) //	35 ± 5 (4) //

[a] Inhibitory antibodies were added to WP suspensions either immediately prior to, or after a 10 minute pre-incubation period with thrombin (POST), then transferred to the microcouette for shearing.

[b] In order to simplify presentation, values presented for % Iα_G for vWF and TSP were corrected for any inhibition obtained with control antibodies. Results were pooled as described in the Methods section.

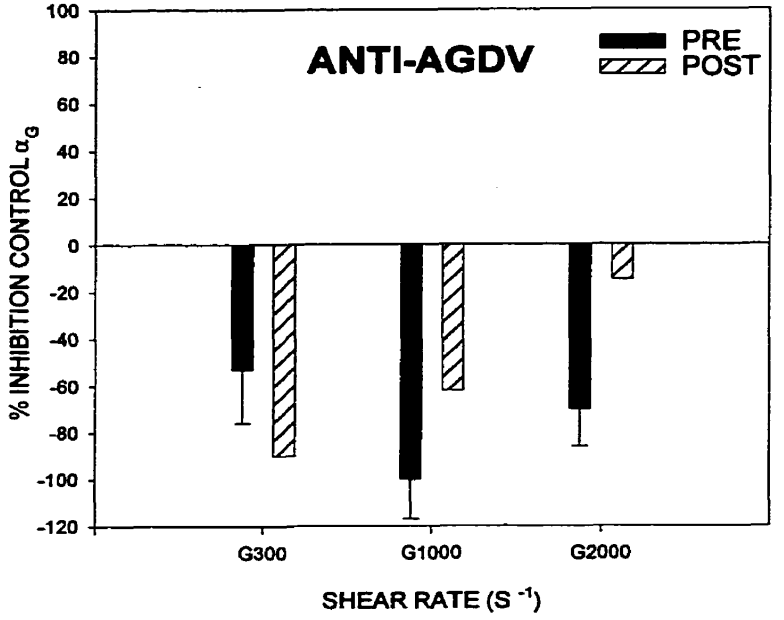
[c] Numbers in parentheses represent numbers of separate experiments

§ $P < 0.05$

// non-significant

3.2.2. Pre-activation of WP with thrombin, τ_{10} ; Role of Fg: Using polyclonal $F(ab')_2$ fragments against Fg as well as a mAb against the γ-carboxyl terminus of Fg (mAb 4A5), shown to be critical for soluble Fg-mediated cross-bridging of platelets in flow (13), we investigated the contribution of surface-expressed Fg to the aggregation of thrombin-activated WP. The mAb 4A5 did not inhibit α_G at any G tested (Fig. 5-4a), whether added prior to or after pre-incubation with thrombin, suggesting that Fg does not directly cross-bridge platelets under these conditions. In fact, mAb 4A5 potentiated α_G by 15 - 100 %. It was therefore surprising to find that polyclonal $F(ab')_2$ fragments against Fg significantly inhibited α_G at all shear rates when added before pre-incubation with thrombin (Fig. 5-4b), with inhibition at $G = 300\ s^{-1}$ significantly greater than at the two higher shear rates ($P < 0.001$). Addition of this antibody after pre-activation with thrombin (verified to bind platelets when added at τ_{10} with less than 20 % decrease in numbers bound than when added at τ_0 ; results not shown), caused no inhibition of α_G at any G, but rather a shear-rate independent potentiation of α_G as we had observed with mAb 4A5. Thus, Fg appeared to be required, but in a role distinct from that of direct cross-bridging of platelets.

a.



b.

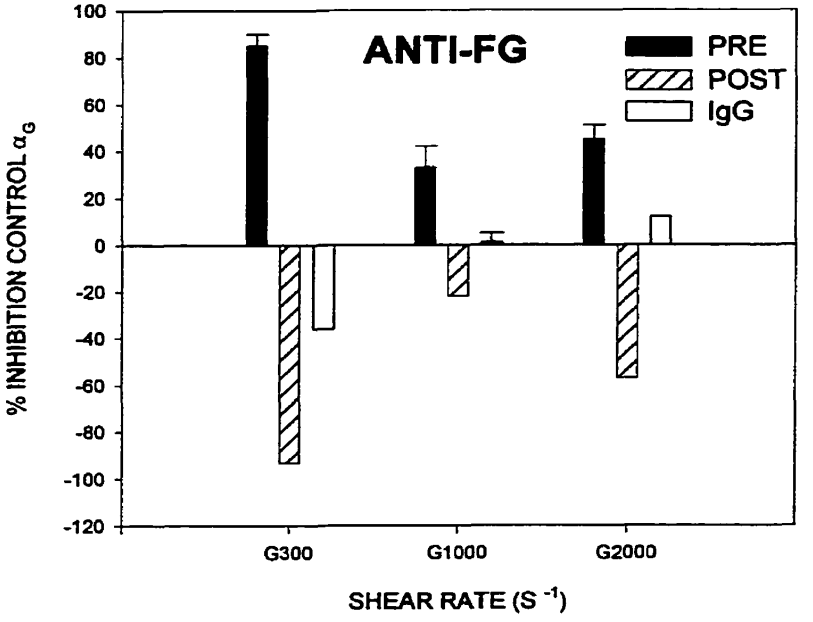


Figure 5- 4. Inhibition of shear-associated aggregation by Fg antagonists in WP pre-activated with thrombin (τ_{10}). WP (4 x 10^4 /μl) were activated with thrombin. A mAb (anti AGDV)(a.), or polyclonal $F(ab')_2$ fragments against Fg (1.3 μM) (b.), were added to WP either immediately prior to or after a 10 minute incubation with thrombin, then sheared. Results shown are represented as the mean ± SEM, and are from 2-9 donors.

3.2.3. Inhibition of adhesive ligands, τ_0: In contrast to the lack of inhibition by mAb 4A5 when platelets were pre-activated with thrombin (τ_{10}), both mAb 4A5 and polyclonal F(ab')$_2$ fragments against Fg significantly inhibited α_G in a shear-rate dependent manner when platelets were activated and immediately sheared (τ_0)(Fig. 5-5), suggesting a direct role for Fg in cross-bridging of platelets in flow at τ_0, especially important at the lowest shear rate. Antibodies against vWF and TSP also inhibited α_G (Fig. 5-5). The inhibition was similar to that observed when added prior to pre-incubation (τ_{10}), except that the % I of α_G by the anti-vWF antibody was significantly lower at G = 1000 s^{-1} ($P < 0.01$), with the minimal % I α_G still close to 50%.

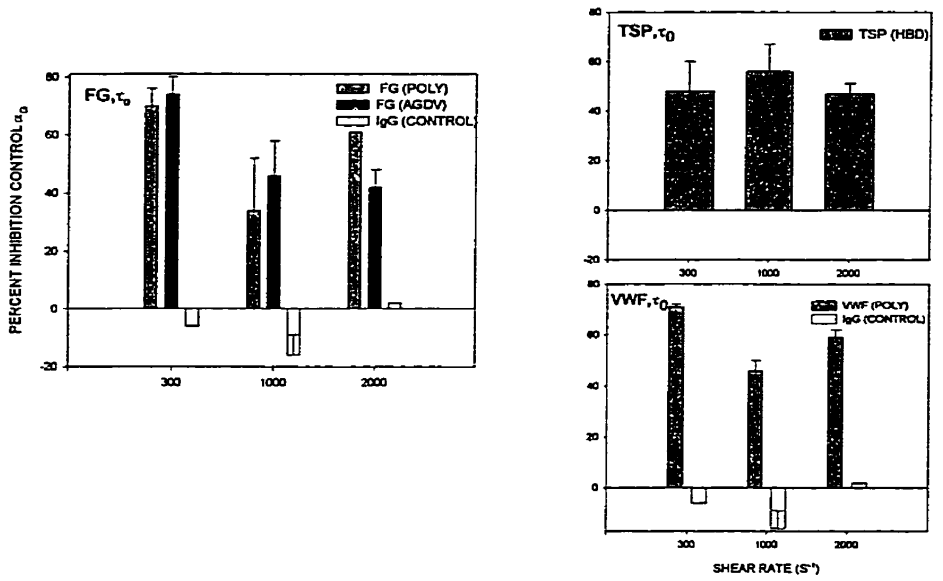


Figure 5-5. Inhibition of shear-associated aggregation by antagonists of Fg, TSP and vWF in WP activated with thrombin and immediately transferred to the microcouette for shearing (τ_0).Washed platelets were activated as in Fig. 4, and sheared in the presence of inhibitors without a pre-incubation period. Inhibitory antibodies used were as in Fig. 3 and 4., as well as polyclonal F(ab')$_2$ fragments against TSP (67 nM) (anti-TSP data were pooled as explained in Table 1). Results shown for inhibition of control α_G (no inhibitor) are from experiments with 3-4 donors.

3.2.4. GPIb and GPIIb-IIIa participation: The mAb 10E5 against the binding pocket of GPIIb-IIIa completely blocked the shear-associated aggregation of WP, at all shear rates and activating conditions (data not shown). The antibody against GPIb (mAb 6D1) caused shear-dependent inhibition at τ_{10}, with greater inhibition (66 ± 7 %) (n = 3) at G = 1000 s^{-1} than at G = 300 or 2000 s^{-1} ($P < 0.01$), where % I α_G were lower (18 ± 3 % (n = 3) and 31 ± 4 % (n = 3) respectively($P < 0.05$)). There was no significant difference between the % I α_G obtained whether mAb 6D1 was added prior to, or after a 10 minute incubation with thrombin. At τ_0, inhibition by mAb 6D1 was surprisingly > 80% at all shear rates studied. This was significantly greater than that at τ_{10} at G = 300 or 2000 s^{-1} (paired t-test $P < 0.05$)(data not shown).

3.3. Investigation of fibrin formation:

Fibrin formation and participation in platelet aggregation were hypothesized based on differences in Fg antagonism observed at the different experimental conditions tested. Accordingly, we used a fluorescently-labelled antibody against Bß 15-42 on fibrin II (FITC-T2G1) to compare binding to WP activated with 0.2 μM PMA which lacks proteolytic activity, versus threshold thrombin concentrations. Thrombin activated platelets clearly showed a 12-fold increase in Fl_{tot} for FITC-T2G1 (Fig. 5-6) over background levels, indicating fibrin formation, while platelets activated with PMA showed only a 1.5-fold increase. Binding of FITC-T2G1 to thrombin-activated WP for only 10 minutes (corresponding to τ_{10} in shear experiments), still yielded 50 % of the maximal binding obtained in 30 minute incubations. These data suggest a potential role for fibrin in the shear-

associated aggregation of thrombin pre-activated platelets.

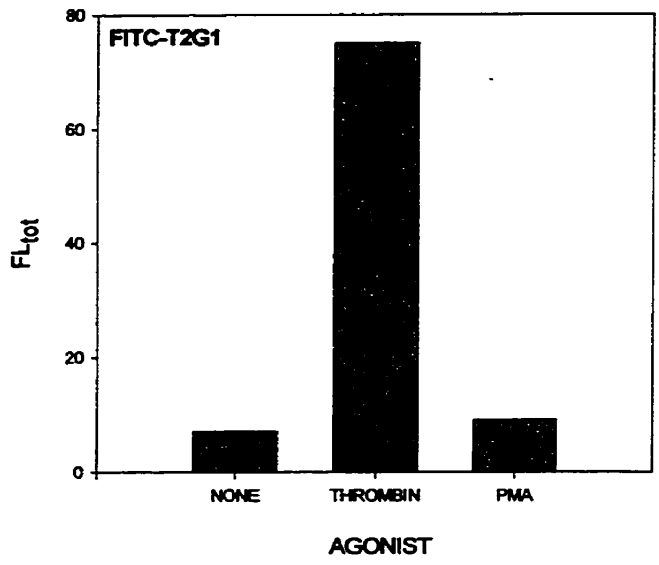


Figure 5-6. Detection of fibrin formation on surfaces of washed platelets activated with threshold thrombin concentrations. Washed platelet suspensions ($10^4/\mu l$) were activated with thrombin, or with 0.2 μM PMA. Labelled antibody was added immediately, then incubated for 30 minutes. Thrombin cleavage dependent production of fibrin-II was detected by mAb FITC-T2G1. Results presented are for the fluorescence (Fl_{tot}) in the presence and absence of agonist, for a representative donor, and are similar to results obtained with all three donors tested.

4. DISCUSSION:

Antibodies against the three adhesive ligands tested, vWF, TSP and Fg, inhibited microaggregate formation by platelets activated with threshold concentrations of thrombin in shear flow. However, the apparent contribution of each ligand, as determined by inhibition of α_G by specific antibodies, varied with shear rate and with the activating conditions preceding shearing.

4.1. Platelet aggregation of WP pre-activated with thrombin for 10 minutes (τ_{10}):

In the aggregation of platelets pre-activated with thrombin (τ_{10}), (i) as in our previous studies at $G = 1000\ s^{-1}$ (20,21), vWF appears to play an important direct role in cross-bridging

of platelets in flow, as determined by a 63 - 75 % inhibition of α_G by a polyconal anti-vWF Ab, added after surface presentation of proteins was complete; (ii) there is a requirement for TSP for optimal microaggregation, with TSP fulfilling dual functions, as determined by differences in inhibition by TSP antibodies added before or after pre-incubation with thrombin: (a) directly contributing to capture of platelets in flow, and (b) a thus far unclarified function, (iii) native Fg does not appear to directly cross-bridge platelets, as evidenced by the complete lack of inhibition by mAb 4A5 added pre or post thrombin, and by the selective inhibition by the polyclonal $F(ab')_2$ anti-Fg fragments, seen only if added prior to thrombin addition; (iv) fibrin is formed on the surfaces of platelets activated with thrombin for 10 minutes, and may participate in platelet aggregation; and (v) glycoprotein GPIIb-IIIa is absolutely required at all shear rates, while there is a shear-dependent requirement for GPIb.

Differences between inhibition of α_G by inhibitory antibodies when added before or after incubation with thrombin, suggest a model where at all shear rates tested vWF is the principal cross-bridging agent, with a cross-bridging component attributable to TSP. Although native Fg does not appear to directly cross-bridge platelets, Fg in its proteolysed form, fibrin, may be important for platelet aggregation at τ_{10}. We hypothesize that $F(ab')_2$ fragments against Fg, can interfere with an early event, such as (i) blocking sites on Fg required for stabilization of Fg or Fn by TSP (17), or (ii) inhibition of fibrin formation and or polymerization. Thus, inhibitory antibodies may disrupt complex formation between ligands, or may block sites required for cross-bridging, where associations between vWF and TSP (15), or Fg and TSP (9,17) may provide stabilization for either partner for actual cross-bridging. We have not ruled out the possibility that the proteolyzed form of Fg, Fn, may

directly cross-bridge platelets via the GPIb-vWF-fibrin axis, or via GPIIb-IIIa by cross-bridging sites other than epitopes targeted by mAb 4A5 or $F(ab')_2$ anti Fg. This would compare with recent results described for normal clot retraction, shown to occur independently of Fg sites involved in platelet binding and aggregation (33). Indeed, a fibrin-associated epitope has been described on gamma (34), which is also implicated in defective mediation of platelet adhesion to immobilized Fg having mutations or deletions in this region (35).

4.2. Platelet aggregation of WP pre-activated with thrombin, τ_0:

In contrast to results obtained at τ_{10}, in thrombin-activated WP suspensions sheared immediately (τ_0), Fg participated directly in the cross-bridging of platelets in flow at all shear rates, as determined by the inhibition of α_G by mAb 4A5. vWF and TSP continued to be required for optimal microaggregation. This is consistent with a model where activation with thrombin at threshold concentrations, such as may occur in the interval preceding amplified thrombin generation, yields platelet subpopulations with submaximal to maximal surface-expression of adhesive ligands, and limited numbers of activated GPIIb-IIIa (20,21), thereby necessitating the composite contribution of all three adhesive ligands for optimizing platelet aggregation. This is paralleled by initial processes in adhesion, where multiple ligands and receptors participate (1).

In our study at τ_0, Fg appears to cross-bridge platelets directly at $G = 2000\ s^{-1}$. Although studies of platelet adhesion to immobilized Fg suggest that the efficiency of

platelet adhesion to Fg drops off by G = 800 s^{-1} (36), studies of platelet aggregation in suspension (29,31) indicate a modest but non-negligible role for Fg at higher G, with a role for Fg in thrombus stabilization postulated (37). TSP appears to contribute to the capture of platelets in shear flow, as in studies of platelet adhesion to immobilized Ca^{2+}-replete purified TSP (38). However, in suspension, this role appears to be shear rate independent.

4.3. The role of vWF at low shear:

Our results indicate a significant requirement for vWF at threshold thrombin concentrations (0.02 - 0.05 U/ml) both at τ_0 and τ_{10}, even at the very lowest shear rate (300 s^{-1}), where soluble Fg is primarily expected to cross-bridge platelets. This is supported by studies where the vWF-GPIb axis was found to be important for thrombus growth at G = 300 s^{-1}, even in the presence of Fg (37). It is however in contrast to the limited role reported for vWF, where normal aggregation was observed in vWF deficient or vWF-antagonized platelet rich plasma activated with ADP or epinephrine (14,39). High agonist concentrations used in these studies would increasingly upregulate platelet activation responses, and would maximally elevate the fraction of the platelet population showing increased activated GPIIb-IIIa receptors and surface-expressed proteins, thereby affecting receptor ligand requirements for cross-bridging. We previously described such differences in platelet activation responses when using intermediate (0.2 U/ml) versus low ($\leq$ 0.05 U/ml) thrombin concentrations (21). In fact, in our current studies, we have found that inhibition of α_G by antibodies against TSP and GPIb is almost negligible at a thrombin-activating concentration of 0.2 U/ml.

4.4. Comparison to ADP-mediated aggregation of WP in shear

A previous study of WP activated with ADP, expressing surface-bound vWF and Fg, and immediately sheared (τ_0), showed similar kinetics of early aggregation as for thrombin-activated platelets (τ_{10}) at $G = 1000\ s^{-1}$ (19). An important role was also ascribed to surface-expressed vWF (20), suggesting parallel mechanisms for bond formation between colliding platelets in flow. However, in contrast to shear-associated aggregation induced by ADP, for thrombin, a role can also be ascribed to Fg/Fn. While ADP activates subpopulations of platelets to maximally express activated GPIIb-IIIa receptors, activation with low concentrations of thrombin only yields subpopulations expressing submaximal numbers of activated GPIIb-IIIa receptors (21). Thus, although vWF still appears to be the principal cross-bridging agent, sites to which vWF bridges on threshold thrombin activated WP may now include TSP and/or fibrin, required to increase numbers of "receptor sites" to yield similar kinetics. Indeed, the vWF-GPIb axis has been shown to play an important role in platelet-fibrin interactions (16,40).

4.5. Concluding remarks:

Intrinsic chemical or physical properties of ligands and receptors are expected to determine the association between varying ligand-receptor combinations. We have shown that interactions between specific pairs of ligands and receptors may be favoured for participation in platelet aggregation, under differing shear and activation conditions. This may further depend on stabilizing complexes formed between alternate pairs, where ligands may additionally function as "receptors". In the clinical setting, treatment for cardiovascular

disease requires that a balance be re-established between anti-coagulant and pro-coagulant systems. Our results indicate that the multiple roles of ligands under varying conditions, may allow mild, multitarget prophylactic treatments, which should help reduce bleeding tendencies while controlling undue thrombus growth. Further studies will be required to link specific epitopes with function for adhesive ligands and receptors in platelet aggregation under varying conditions, and to define targets for use in interventional therapy.

5. ACKNOWLEDGMENTS

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6. REFERENCES:

1. Savage B, Almus-Jacobs F, Ruggeri ZM. Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. Cell 1998; 94: 657-66.

2. Suzuki H, Kinlough-Rathbone RL, Packham MA, Tanoue K, Yamazaki H, Mustard JF. Immunocytochemical localization of fibrinogen during thrombin-induced aggregation of washed human platelets. Blood 1988; 71: 1310-20.

3. Hanson SR, Harker LA. Interruption of acute platelet-dependent thrombosis by the synthetic antithrombin D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone. Proc Natl Acad Sci USA 1988; 85:3184-8.

4. Roald HE, Sakariassen KS. Axial dependence of collagen-induced thrombus formation in flowing non-anticoagulated human blood. Anti-platelet drugs impair thrombus growth and increase platelet-collagen adhesion. Thromb Haemost 1995; 73:126-31.

5. Yamamoto H, Vreys I, Stassen JM, Yoshimoto R, Vermylen J, Hoylaerts MF. Antagonism of vWF inhibits both injury induced arterial and venous thrombosis in the hamster. Thromb Haemost 1998; 79: 202-10.

6. Bini A, Fenoglio JJ Jr, Mesa-Tejada R, Kudryk B, Kaplan KL. Identification and distribution of fibrinogen, fibrin and fibrin(ogen) degradation products in atherosclerosis. Arteriosclerosis 1989; 9: 109-21.

7. Wight TN, Raugi GJ, Mumby SM, Bornstein P. Light microscopic immunolocation of thrombospondin in human tissues. J Histochem Cytochem 1985; 33: 295-302.

8. Leung LLK. Role of thrombospondin in platelet aggregation. J Clin Invest 1984; 74: 1764-72.

9. Legrand C, Thibert V, Dubernard V, Begault B, Lawler J. Molecular requirements for the interaction of thrombospondin with thrombin-activated platelets: modulation of platelet aggregation. Blood 1992; 79: 1995-2003.

10. Tollefsen DM, Majerus PW. Inhibition of human platelet aggregation by monovalent antifibrinogen antibody fragments. J Clin Invest 1975; 1259-68.

11. Legrand C. Dubernard V, Rabhile-Sabile S, Morandi da Silva V. Functional and clinical significance of thrombospondin. Platelets 1997; 8: 211-23.

12. Beacham DA, Wise RJ, Turci SM, Handin RI. Selective inactivation of the arg-gly-asp-ser (RGDS) binding site in von Willebrand factor by site-directed mutagenesis. J Biol Chem 1992; 267: 3409-15.

13. Liu Q, Matsueda G, Brown E, Frojmovic MM. The AGDV residues on the gamma chain carboxyl terminus of the platelet-bound fibrinogen are needed for platelet aggregation. Biochimica Biophysica Acta 1997; 1343: 316-26.

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

15. Barabino GA, Wise RJ, Woodbury VA, Zhang B, Bridges KA, Hebbel HP, Lawler J, Ewenstein BM. Inhibition of sickle erythrocyte adhesion to immobilized thrombospondin by von Willebrand factor under dynamic flow conditions. Blood 1997; 89: 2560-7.

16. Lozcalzo J, Inbal A, Handin RI. Von Willebrand protein facilitates platelet incorporation in polymerizing fibrin. J Clin Invest 1986; 78: 1112-9.

17. Leung LLK, Nachman RL. Complex formation of platelet thrombospondin with fibrinogen. J Clin Invest 1982; 70: 542-9.

18. Rabhile-Sabile S, Thibert V, Legrand C. Thrombospondin peptides inhibit the secretion-dependent phase of platelet aggregation. Blood Coag and Fibrinolys 1996; 7: 237-40.

19. 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.

20. Kasirer-Friede A, Frojmovic MM. Ristocetin and thrombin-induced platelet aggregation at physiological shear rates: Differential roles for GPIb and GPIIb-IIIa receptor. Thromb Haemost 1998; 80: 428-36

21. Kasirer-Friede A, Legrand C, Frojmovic MM. Thrombin receptor occupancy modulates aggregation efficiency and platelet surface expression of von Willebrand factor and thrombospondin, at low thrombin concentrations. Thromb Haemost 1999, in press

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

23. Goto S, Salomon DR, Ikeda Y, Ruggeri ZM. Characterization of the unique mechanism mediating the shear-dependent binding of soluble von Willebrand factor to platelets. J Biol Chem 1995; 270:23352-61.

24. Matsueda GR, Bernatowicz. Characterization of a monoclonal antibody that binds to the carboxyl-terminus of the fibrin gamma-chain In: M.W. Mossesson et al. (Eds.), Fibrinogen 3. Biochemistry, Biological functions, Gene Regulation and Expression, Proceedings of the International Fibrinogen Workshop, Elsevier, Milawaukee, WI, 1988: 133-36.

25. Lawler J, Derick LH, Connoly JE, Chen JH, Chao FC. The structure of human thrombospondin. J Biol Chem 1985; 260: 3762-72.

26. Legrand C, Dubernard V, Kieffer N, Nurden AT. Use of a monoclonal antibody to measure the surface expression of thrombospondin following platelet activation. Eur J Biochem 1988;171: 393-9. 27. Legrand C, Pidard D, Beiso P, Tenza D, Edelman L. Interaction of a monoclonal antibody to glycoprotein IV (CD36) with human platelets and its effects on platelet function. Platelets 1991; 2: 99-105.

28. Kudryk B, Rohoza A, Ahadi M, Chin J, Wiebe ME. Specificity of a monoclonal antibody for the NH2 -terminal region of fibrin. Molec Immunol 1984; 21: 89-94.

29. Goldsmith HL, Frojmovic MM, Braovac S, McIntosh F, Wong,T. Adenosine diphosphate- induced aggregation of human platelets in flow through tubes: III. Shear and extrinsic fibrinogen-dependent effects. Thromb Haemost 1994; 71: 78-90.

30. Xia Z, Wong T, Liu Q, Kasirer-Friede A, Brown E, Frojmovic MM. Optimally functional fluorescein isothiocyanate-labelled fibrinogen for quantitative studies of binding to activated platelets and platelet aggregation. Br J Haematol 1996; 93: 204-14.

31. Xia A, Frojmovic MM. Aggregation efficiency of activated normal or fixed platelets in a simple shear field: Effect of shear and fibrinogen occupancy. Biophys J 1994; 66: 2190-2201.

32. Tang SS, Frojmovic MM. The effects of pCO_2 and pH on platelet shape change and aggregation for human and rabbit platelet-rich plasma. Thromb Res 1977; 10: 135-45.

33. Rooney MM, Farrell DH, van Hemel BM, de Groot PG, Lord ST. The contribution of the three hypothesized integrin-binding sites in fibrinogen to platelet-mediated clot

retraction. Blood. 1998; 92: 2374-81.

34. Schielen WJG, Adams HPHM, van Leuven K, Voskuilen M, Tesser GJ, Nieuwenhuizen W. The sequence gamma-(312-324) is a fibrin specific epitope. Blood 1991; 77: 2169-73.

35. Sixma JJ, van Hemel BM, Galanakis DK, Rooney MR, Scharrer I, de Groot PG. Mutations in the gamma chain of fibrinogen between γ315 and γ322 are associated with an absence of platelet adhesion under conditions of flow. Blood 1998; 92(Suppl): Abstract 1432.

36. Savage B, Salvidar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. Cell 1996; 84: 289-97. .

37. Ruggeri ZM, Savage B, Dent JA, Salvidar E. Synergistic adhesive interactions supporting platelet adhesion and aggregation under flow. Blood 1998; 92(Suppl):Abstract 1424.

38. Agbanyo FR, Sixma JJ, de Groot PG, Languino LR, Plow EF. Thrombospondin-platelet interactions: Role of divalent cations, wall shear rate, and platelet membrane glycoproteins. J Clin Invest 1993; 92: 288-96.

39. Goto S, Ikeda Y, Saldivar E, Ruggeri ZM. Distinct mechanisms of platelet aggregation as a consequence of different shearing flow conditions. J Clin Invest 1998; 101:479-86.

40. Endenburg SC, Hantgan RR, Lindeboom-Blokzijl L, Lankhof H, Jerome WG, Lewis JC, Sixma JJ, de Groot PG. On the role of von willebrand factor in promoting platelet adhesion to fibrin in flowing blood. Blood 1995; 86: 4158-65.

Chapter 6

SUMMARY OF RESULTS and GENERAL DISCUSSION

1. ORIGINAL CONTRIBUTIONS AND SUMMARY OF RESULTS

We have evaluated interactions between metabolically active platelets at well-defined shear rates, for a series of agonists. Most previous studies examined platelet aggregation at low, undefined shear flow conditions (G estimated to be $<100\ s^{-1}$)[1,2] and often at high agonist activating concentrations, or at very high shear rates ($G \geq 6000\ s^{-1}$) in the absence of chemical agonist[3,4]. We have attempted to more closely adhere to physiologic ranges of shear rate, namely ~100 - 2500 s^{-1}. We have also emphasized the importance of determining receptor-ligand interactions at low agonist concentrations (2 - 5 μM ADP, $\leq$0.05 U/ ml thrombin), as these are expected to most truly reflect concentrations available in vivo upon initiation of physiologically relevant processes of hemostasis and thrombosis[5].

Our focus was on the role of von Willebrand factor (vWF) in mediating platelet aggregation induced by varying concentrations of platelet activators, and at varying shear rates. The picture emerges of vWF as a versatile, extremely sticky adhesive protein. It appears to be especially important at low agonist activating concentrations, where conditions for effective platelet capture in flow are expected to be the most stringent, due to submaximal responses in several platelet parameters. Thus, we have been the first group to explore specifically the kinetics and capture efficiencies of platelet shear-associated aggregation mediated by soluble vWF bound to GPIb, in the presence of the chemical modulator, ristocetin. We demonstrated that the efficiency attained was eight-fold greater than for Fg-mediated aggregation under similar shear-flow conditions[6], and was dependent on the platelet GPIb receptor alone. Furthermore, as few as10 molecules, or fewer, of bound vWF were sufficient to cross-link platelets. (Chapter 3).

Platelets activated with the physiologic agonists ADP and thrombin, showed increased surface expression of von Willebrand factor following activation, in the absence of any externally added soluble ligands. Initial studies established the existence of a threshold thrombin concentration which caused a fraction of the platelet population to maximally express vWF. This threshold concentration occurred with an EC_{50} (effective concentration causing 50% of maximal changes) identical to that for a similar threshold response for the onset of platelet aggregation. Additionally we showed that this EC_{50} was significantly lower than that for the expression of surface P-selectin or activated GPIIb-IIIa receptors, suggesting uncoupling of platelet activation responses. Unlike shear-associated aggregation in the presence of a specific modulator of the vWF-GPIb interaction, ADP-activated washed platelets showed a shear-sensitive role for GPIb and an unconditional and total requirement for activated GPIIb-IIIa. Thus, rapid initial capture of platelets via surface-bound vWF on one platelet, and GPIb on another platelet, insufficient to mediate aggregation alone, could nevertheless extend contact times between colliding platelets to allow stable bond formation to occur via activated GPIIb-IIIa. Aggregation induced by low thrombin concentrations in platelets pre-activated with thrombin for 10 minutes prior to shearing (τ_{10}), showed an absolute requirement for vWF, and similar glycoprotein receptor participation and efficiencies of platelet capture as when induced by ADP. However, this similarity occurred despite underlying differences in participation by the ligands TSP and Fg.

We were the first to demonstrate that TSP could mediate shear-associated microaggregation of platelets pre-activated with threshold concentrations of thrombin, both by cross-bridging platelets and by a second, as yet undefined role. We also clearly showed

that under these conditions, native Fg does not directly cross-bridge platelets, but that in the thrombin-proteolyzed form, fibrin, it may either directly link platelets through as yet undefined epitopes, or it may function as a "receptor", thereby providing additional sites to which vWF, surface-expressed on another platelet, may cross-bridge.

Platelets activated by thrombin and transferred immediately to the microcouette for shearing (τ_0), relied on different ligand-receptor combinations for effecting aggregation. In this case, native Fg directly cross-bridged platelets in a shear-dependent manner, in addition to a partial reliance on TSP. A much higher than previously suggested role was ascribed to the GPIb receptor at all shear rates. This, together with the fact that under all conditions tested, inhibition of vWF caused between 63-98 % inhibition of microaggregation, suggests that the vWF-GPIb axis may be supremely important for direct capture and initial cross-bridging of platelets in minimally activated platelets.

1.1. Thrombin concentration-dependent platelet responses

Using thrombin antagonists, we showed that requirements for thrombin occupancy of its platelet receptors varied with thrombin concentration. Previous studies had suggested that for platelet aggregation induced by low concentrations of thrombin, occupancy of its receptors was required only in the first minute or so following activation to allow secretion to occur[7,8]. However, we showed that for the initiation of aggregation, sustained occupancy of thrombin receptors by proteolytically active thrombin was required for maintenance of surface-expressed vWF, TSP and likely for appropriate activated GPIIb-IIIa conformation. We also demonstrated that following thrombin neutralization, the presence of soluble Fg pre-bound to activated GPIIb-IIIa, was insufficient for mediation of aggregation, even at flow

conditions optimal for Fg.

1.2 Caveats

The above must be considered with the following caveats. 1) Our experiments were performed in a closed system, rather than in a "one-pass" system such as exists in the vasculature. Thus, cells were exposed to a constant agonist concentration (no dilution effects) and shear rate (normally varying throughout the circulation) during the duration of each experiment. However, short times were used in actual aggregation measurements, with initial rates estimated for "zero" time, with the first experimental time point being 10 seconds. 2) Uniform laminar shear was applied in contrast to the pulsatile shear stresses to which blood is exposed within the vasculature. 3) We have used isolated platelets with selective re-constitution of plasma proteins and the absence of additional blood or vessel wall cells, which can contribute further activators (neutrophil cathepsin G [9]) or inhibitors (EC nitric oxide, prostacyclin[10]). 4) The transfer time between the Eppendorf tubes used to pre-mix platelet suspensions with agonists and inhibitors, and the microcouette device used for shearing, also added 20-30 seconds prior to shearing. This activation time is longer than would be expected in normal physiology, where adhesive events occur within milliseconds. However, as flow greatly increases the flux of platelets, at the short times tested ($\leq$60 seconds), aggregation becomes significant only under dynamic conditions, and cross-bridging partners are expected to validly reflect in vivo associations occurring at given shear rates. In the pathological state, there may be more chronic exposure to agonists, abrogating this caveat, and modeled by our studies at τ_{10}.

2. DISCUSSION

2.1. Chemical and physical determinants of platelet aggregation

The interplay between chemical and physical modulators of platelet aggregation may be illustrated by the observations of Dr. Goldsmith et al[11]. They found that washed platelets aggregated in tube flow only when minimally activated (0.7 μM ADP), and were subjected to average wall shear rates of G $\geq$300 s^{-1}, with both conditions simultaneously required, and no aggregation observed at G $\leq$ 100 s^{-1}. A synergism between flow and activation was also described by Goto et al[12], for the agonist epinephrine. Our results reflect the modulator effect of these two elements at the molecular level. Chemical activation can directly upregulate GPIIb-IIIa affinities, induce the release of alpha granule-stored adhesive ligands vWF and Fg, already present in plasma, as well as of new ligands like TSP, and of the dense granule-stored activator, ADP. Physical factors regulate the frequency of collisions[13], contact times[14], energies of collision (transfer of kinetic energy to energy of bond formation), and can directly alter the quaternary structure of molecules, as reported for shear-induced unfolding and extension of surface-bound vWF[15].

2.2 Platelet cross-bridging receptor sites

Increases in ADP concentration used to activate platelet-rich plasma suspensions, yield increasing fractions of platelets expressing maximal numbers of activated GPIIb-IIIa receptors (% P^*), with % P^* corresponding to % platelets incorporated into platelet aggregates[16,17]. In contrast to this, increases in thrombin concentrations used to activate washed platelets, leads to a graded response in both the fraction of platelets expressing activated GPIIb-IIIa, and in the actual numbers of receptors which are activated (Fig. 6-1).

The percentage of platelets incorporated into aggregates is however much greater than this number. At threshold thrombin activating concentrations, the low numbers of GPIIb-IIIa* available may limit the potential of ligands to capture or to completely link platelets in flow via this receptor, since (i) fewer GPIIb-IIIa* may require more collisions before effective capture can occur, or (ii) fewer cross-bridging sites may reduce avidity, which may be needed to maintain effective cross-bridging. A similar graded response occurs for TSP surface expression as well.

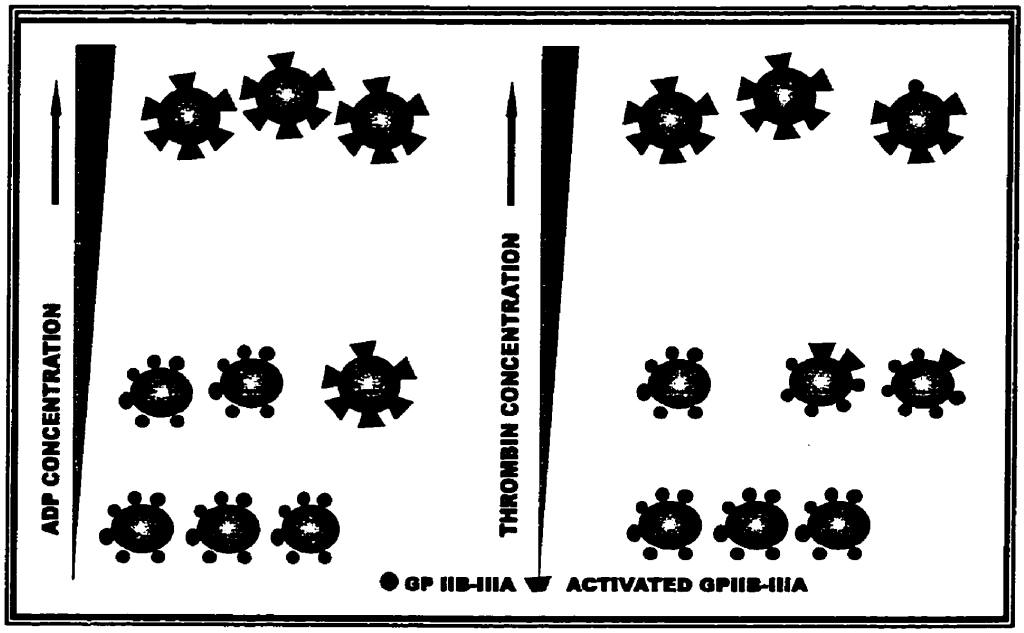

Fig. 6-1. Glycoprotein (GP) IIb-IIIa receptor activation as a function of agonist activation. Although at high agonist concentrations, both ADP and thrombin will produce activation of mostly all GPIIb-IIIa receptors on the entire platelet population, at low concentrations, a different pattern of GPIIb-IIIa activation occurs. Whereas activation at such concentrations with ADP will produce a subpopulation of platelets with all their GPIIb-IIIa receptors fully activated, thrombin activation results in subpopulation responses, with only a fraction of receptors on each platelet being activated.

The greater ability of thrombin than of ADP to drive secretion[18], its catalytic function, and differences between ADP and thrombin signaling pathways, however, can provide additional molecules for cross-bridging of platelets activated with thrombin, each associated with their respective receptors. There are at least two different mechanisms which may compensate for low activated GPIIb-IIIa density (numbers per platelet) on platelet subpopulations (Fig. 6-2). In the first case, called the mad dash model (τ_0), for platelets activated and sheared immediately, corresponding to normal physiological processes, the composite contribution of ligands Fg, vWF and TSP, and receptors GPIb and GPIIb-IIIa*, is required to effect aggregation, with Fg participating in a direct cross-bridging role. In a pathological model corresponding to liberation of activated platelets from entrapped sites with low levels of thrombin generation[19], called the chronic evolution model (τ_{10}), platelets may aggregate, by way of vWF, TSP and fibrin (Chapter 5), with both direct participation in aggregation and a secondary role postulated for TSP. GPIb is less important than in the mad dash model (τ_0), and GPIIb-IIIa* is absolutely required. Again, a multi-component model is expected to compensate for limited GPIIb-IIIa* availability. Glycoprotein Ib as a receptor remains important under these conditions, since approximately 25,000 receptors per platelet are available on the surface of each platelet in the suspension, with a minimum of 20,000 receptors competent to bind vWF in the presence of ristocetin. Very little loss due to internalization is expected at concentrations of $\leq$0.05 U/ml thrombin[20,21]. This may be compared with approximately 6 -10,000 molecules per platelet on $\leq$20 % of the population for GPIIb-IIIa*, or an estimated 10,000-20,000 antibody-accessible fibrin molecules on 40-50% of platelets (with GPIb also required for vWF-mediated platelet-fibrin interactions).

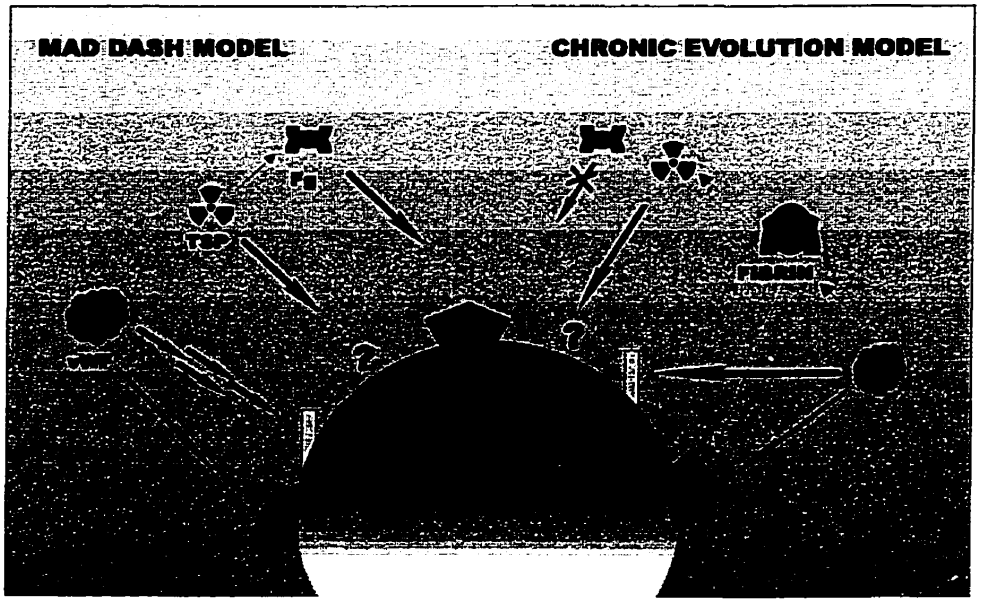

Fig. 6-2. Models for participation of adhesive ligands and glycoprotein receptors in thrombin-induced shear-associated aggregation. When platelets are activated with threshold thrombin concentrations and immediately placed in a shear field, vWF, TSP and Fg appear to directly participate in mediating the aggregation (mad dash model; τ_0). GPIb and GPIIb-IIIa are both extremely important at all shear rates. When platelets are pre-activated with thrombin for 10 minutes prior to shearing (chronic evolution model), native Fg is no longer required, but when proteolysed to fibrin, is now needed either to cross-bridge platelets through non AGDV sites, or to function as a "receptor" for cross-bridging to, by vWF or TSP.

2.3 Role of von Willebrand Factor

VWF is competitively at an advantage over Fg in our "chronic evolution" model, as it could theoretically cross-bridge to four distinct "receptors" GPIb, GPIIb-IIIa*, TSP or fibrin (discussed in Chapter 1, section 4.4). As suggested by our studies, the vWF-GPIb axis likely

acts in a facilitative role, bringing cells together to allow further bond formation between additional receptor-ligand partners such as Fg-GPIIb-IIIa*, which may be further stabilized by TSP[22]. Thus, multi-component adhesions would provide the greatest strength of platelet-platelet cohesion.

Quantitative differences in the surface expression of adhesive proteins and activated GPIIb-IIIa receptors, and qualitative differences in receptor-ligand participation in aggregation, varied with the activation thrombin concentration (Chapter 3,4). The dependence of platelet aggregation on GPIb and TSP at intermediate shear rates decreased with increasing thrombin concentration for thrombin pre-activated platelets. At higher agonist concentrations, Fg may be of primary importance for cross-bridging at shear rates below G ~ 500 [3,23], due to a plasma concentration advantage and full activation of its binding partner, GPIIb-IIIa*. vWF should gain importance again as shear stresses increase and more severely limit contact times available for molecular orientation for bond formation during cross-bridging. Recent studies by Ruggeri et al[24], 1998, suggest that both Fg and vWF may function over a wider range of shear rates than previously believed[3]. At lower shear rates (G = 300 s^{-1}), and in the presence of plasma concentrations of Fg, vWF may be important for thrombus growth in a non-pre-activated system, while Fg may stabilize aggregates at G up to 1500 s^{-1}[24]. In suspension, for platelets pre-activated as in our experiments, vWF was found not to be important in mediating platelet aggregation[25]. This however is not necessarily in disagreement with our results, and merely cautions as to the sensitivity of data to slight changes in experimental conditions. In their studies, surface expression of adhesive proteins was not determined, and full activation of platelets was likely induced at the much higher

agonist concentrations used (20 μM ADP and 20 μM epinephrine), thereby shifting the balance towards different receptor-ligand participation. An essential role for the vWF-GPIb axis has also been demonstrated in platelet adhesion to fibrin, collagen and fibronectin (see Chapter 1, sections 2.1.1, 4.4 and 5.3)), and is therefore of broad physiological importance.

2.4 Other Paradigms:

Initial processes for adhesion have also recently been shown to require multiple receptor-ligand combinations to ensure platelet attachment[26]. An additional paradigm of a multi-component system is that of neutrophil hetero- or homotypic aggregation. As such, in neutrophil-neutrophil interactions, dynamic conditions also dictate molecular bonding partners for mediating aggregation. Thus, at venular shear rates (G = 20 - 200 s^{-1} [27]), neutrophils may aggregate via ß2 integrins (LFA-1 or MAC-1). However, at increasing shear rates (G $\geq$400 s^{-1}), L-selectin and and its receptor PSGL-1 on individual neutrophils are required to mediate initial transient capture, followed by activation and firm capture via ß2 integrins[28-30]. Indeed, activated platelet co-aggregation with neutrophils has also been found to depend on multiple receptor-ligand combinations similar to that for neutrophil homotypic aggregation, but with P- (instead of L-)selectin provided by activated platelets (P^*)[31], and GPIIb-IIIa*-Fg on platelets cross-bridging to CD11b/ or CD11c/ CD18[32]. Thus, intrinsic biomechanical properties of specific receptor-ligand combinations, can limit potentials for interaction at varying shear rates, with in our case, vWF, able to capture platelets under the full range of G from physiological (G $\approx$30-2000 s^{-1}) to pathological (G $\geq$10,000 s^{-1}).

2.5 Reflections on platelet thrombin receptors:

Our results clearly indicate a functional difference between platelet responses when they are activated at thrombin concentrations of ≤0.05 U/ml versus ≥0.2 U/ml. This is reflected by requirements for thrombin receptor occupancy by catalytically active thrombin, for optimal shear-associated aggregation, and for surface expression of vWF and TSP. The sustained requirement for catalytically active thrombin suggests that even at very low concentrations, a proteolytic event may be involved. This is consistent with results by Liu et al[33], where even at low thrombin concentration, cleavage of PAR-1 occurred, and suggests possible cooperativity between high and moderate affinity receptors. Alternatively, allosteric changes in the exosites may be induced by occupation of thrombin by PPACK[34], and may lead to changes in engagement of the receptor for downstream signaling events. The conversion of fibrinogen to fibrin, required for optimal aggregation, may occur with slower kinetics at lower thrombin concentrations. Thus, the abrogation of thrombin catalytic function by its antagonists, may more greatly affect fibrin receptor- or ligand function dependent events mediated by a high-affinity receptor. However, it is difficult to explain how this would cause the ≥ 85 % inhibition of soluble vWF binding to thrombin-activated platelets observed, which is expected to be directly to GPIIb-IIIa*. Therefore, differences in requirements for thrombin receptor occupancy at different thrombin activating concentrations, for platelet aggregation and vWF, and TSP surface-expression, are apparently unrelated to fibrin formation.

2.6 Implications for therapeutics:

Our studies have added to the knowledge of hemostasis by highlighting normal mechanisms which may be involved in platelet aggregation under physiological conditions, and corroborate previously described important roles for the GPIb-axis as well as for activated GPIIb-IIIa. The illustration of platelet aggregation as being dependent on multiple receptor ligand pairs in the earliest stages of hemostatic and thrombotic processes, can provide a basis for "mild" prophylactic therapies targeted towards multiple ligands and receptors. Several antithrombotic agents have been developed against GPIIb-IIIa , with animal and FDA-approved drugs for human use including abciximab (c7E3, Reopro[35,36]), and RGD-related compounds such as tirofiban (Aggrastat[37,38]) and eptifibatide (integrilin[39]). Drugs have been targeted against ADP receptors (ticlopidine and clopidogrel[40]) or directly against thrombin (low molecular weight heparin[41] and hirudin[42]) or its production (F.Xa inhibitors[43]). Although adhesive ligands Fg and vWF[44,45] have also been targeted, TSP antagonism has not been investigated. The appearance of fibrin in atherosclerotic plaques has been correlated with the severity of lesions[46]. Additionally, it has been shown that neutrophil deposition may be increased on fibrin, contributing to inflammatory processes[47]. Our results suggest that fibrin formed on platelet surfaces may participate in platelet aggregation as well. Our discovery of residual inhibition of platelet aggregation by thrombin antagonists at low thrombin activating concentrations despite normal platelet secretion and fibrinogen equilibrium binding, suggests an explanation for the bleeding complications described in some patients[48]. A better understanding of these effects should allow compensatory compounds to be administered which would correct for lost adhesive function while neutralizing the pro-coagulant aspects contributed by thrombin.

3. FUTURE DIRECTIONS:

The release of soluble alpha granule proteins is generally thought to occur in close temporal proximity to the appearance of alpha granule membrane protein P-selectin on platelet surfaces, and as such it is often used as an indicator of secretion from activated platelets. However, studies by Ginsberg et al[49] have suggested that fibronectin, an alpha granule protein, may actually appear on platelet surfaces well in advance of the appearance of soluble granule-derived fibronectin. In our studies, we have shown a disparate increase in the fraction of platelets showing upregulation of surface expression of vWF, than of P-selectin, with vWF expression occurring at lower agonist concentrations, suggesting independent mobilization of this protein from intracellular stores. We have thus far not verified whether platelets with vWF also expressed P-selectin, or if that occurred on a different subset of platelets, and this could easily be done using two-colour fluorescence. Studies by Dr. Goldsmith et al[50], have suggested that the time required for surface organization of various secreted proteins may not be identical. The question raised would tie in well with our experiments, since investigation of temporal appearance on platelet surfaces under a range of conditions would help further define availability of ligands for aggregation. This may need to be checked under flow conditions as well, to take into account post-aggregatory changes which may modulate ligand accessibility.

Further to this, model systems may be used to determine affinities between individual receptor-ligand pairs as a function of receptor availability (GPIb, resting versus activated GPIIb-IIIa, and potentially, TSP and fibrin as receptors) and to relate these to efficiencies of aggregation. Actual numbers of such "receptors" should be taken into account in order to

consider mass-action effects on binding events which must occur in very short periods of time, due to flow diluting out unbound ligands or released agonists.

As our results were procured using washed platelets, in the absence of most plasma proteins or additional cells, reconstitution experiments selectively adding back adhesive plasma proteins, blood cells and cellular pro- and anti-coagulant chemical modulators, should be done to validate the importance of the multi-ligand, multi-receptor model proposed.

We have alluded to a potential role for fibrin in platelet aggregation at threshold thrombin activating concentrations. Decreased platelet adhesion to Fg from patients with mutations in a fibrin neo-epitope[51], at residues 315-322 of the γ-carboxyl terminus of fibrinogen/fibrin[52] has been described. It would be interesting to evaluate this new site for mediation of platelet aggregation in model systems. Similarly, previous studies have not determined the ability of TSP to directly mediate aggregation, while taking into account TSP affinities for its multiple potential platelet receptors (GPIIb-IIIa, IAP, GPIV; see Chapter 1, section 5.2.1.) or for other adhesive ligands (FG, vWF or FN) bound to their respective receptors. These would test functions of TSP other than those for amplification of secretion processes, as previously proposed[53]. These additional studies should provide new data which may prove useful in the development of novel strategies for anti-thrombotic therapies or to improve therapies in bleeding disorders.

4. REFERENCES:

1. Timmons S, Hawiger J. von Willebrand factor can substitute for plasma fibrinogen in ADP-induced platelet aggregation. Trans Assoc Am Phys 1986; 99: 226-35.

2. De Marco L, Girolami A, Zimmerman TS, Ruggeri Z. von Willebrand factor interaction with the glycoprotein IIB/IIIa complex: its role in platelet function as demonstrate in patients with congenital afibrinogenemia. J Clin Invest 1986; 77: 1272-7.

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

4. Goto S, Salomon DR, Ikeda Y, Ruggeri ZM. Characterization of the unique mechanism mediating the shear-dependent binding of soluble von Willebrand factor to platelets. J Biol Chem 1995; 270:23352-61.

5. Shuman MA, Levine SP: Thrombin generation and secretion of platelet factor 4 during blood clotting. J Clin Invest 61: 1102-1106, 1978.

6. Xia A, Frojmovic MM. Aggregation efficiency of activated normal or fixed platelets in

a simple shear field: Effect of shear and fibrinogen occupancy. Biophys J 1994; 66: 2190-2201.

7. Holmsen H, Dangelmaier CA, Holmsen HK. Thrombin-induced platelet responses differ in requirement for receptor occupancy; evidence for tight coupling of occupancy and compartmentalized phosphatidic acid formation: J Biol Chem 1981; 256: 9393-96.

8. Greco, NJ, Tenner Jr. TE, Tandon NN, Jamieson GA. PPACK-Thrombin inhibits thrombin-induced platelet aggregation and cytoplasmic acidification but does not inhibit platelet shape change. Blood 1990; 75:1983-90.

9. Larosa CA, Rohrer MJ, Benoit SE, Rodino LJ, Barnard MR, Michelson AD. Human neutrophil cathepsin G is a potent platelet activator. J Vasc Surgery 1994; 19: 306-19.

10. Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae KR, Hug BA, Schmidt AM, Stern DM. Endothelial cells in physiology and in pathophysiology of vascular disorders. Blood 1998; 91: 3527-61.

11. Goldsmith HL, Frojmovic MM, Braovac S, McIntosh F, Wong,T. Adenosine diphosphate-induced aggregation of human platelets in flow through tubes: III. Shear and extrinsic fibrinogen-dependent effects. Thromb Haemost 1994; 71: 78-90.

12. Goto S, Ikeda Y, Murata M, Handa M, Takahashi E, Yoshioka A, Fujimura Y, Fukuyama M, Handa S, Ogawa S. Epinephrine augments shear-induced platelet aggregation. Circulation 1992; 86: 1859-63.

13. Smoluchowski M von. Versuch einer mathematischen Theorie der Koagulationskinetik kolloider Lösungen. Z Phys Chem 1917; 92: 129-68.

14. Manley, R. St. J., and S.G. Mason. Particle motions in sheared suspensions. II. Collisions of uniform spheres. J. Colloid Sci 1952; 7: 354-69.

15. Siedlicki CA, Lestini BJ, Kottke-Marchant K, Eppell SJ, Wilson DL, Marchant RE. Shear-dependent changes in the three-dimensional structure of human von Willebrand factor. Blood 1996; 88: 2939-50.

16. Frojmovic MM, Mooney RF, Wong T. Dynamics of platelet glycoprotein IIb-IIIa receptor expression and fibrinogen binding. I. Quantal activation of platelet subpopulations varies with adenosine diphosphate concentration. Biophys J 1994; 67: 2060-8.

17. Frojmovic MM, Mooney RF, Wong T. Dynamics of platelet glycoprotein IIB-IIIa receptor expression and fibrinogen binding. II. Quantal activation parallels platelet capture in stir-associated microaggregation. Biophys J 1994; 67: 2069-75.

18. Holmsen H. Platelet secretion and energy metabolism. In Hemostasis and Thrombosis: Basic principles and clinical practice, third ed 1994; Colman RW, Hirsh J, Marder VJ, Salzman EW. J.B. Lippincott Co, Philadelphia.

19. Sevitt S. The structure and growth of valve-pocket thrombi in femoral veins. J Clin Pathol 1974; 27: 517-28.

20. Michelson AD, Benoit SE, Kroll MH, Li JM, Rohrer MJ, Kestin AS, Barnard MR. The activation-induced decrease in the platelet surface expression of the glycoprotein Ib-IX complex is reversible. Blood 1994; 3562-73.

21. Rao GHR, Peller JD, White JG. Influence of ionized calcium on thrombin-induced down regulation of GPIb/IX receptors on human platelets. Thromb Res 1997; 85: 23-31.

22. Leung LLK, Nachman RL. Complex formation of platelet thrombospondin with

fibrinogen. J Clin Invest 1982; 70: 542-9.

23. Savage B, Salvidar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. Cell 1996; 84: 289-97.

24. Ruggeri ZM, Savage B, Dent JA, Salvidar E. Synergistic adhesive interactions supporting platelet adhesion and aggregation under flow. Blood 1998; 92 (SuppI): Abstr #1424.

25. Goto S, Ikeda Y, Salvídar E, Ruggeri ZM. Distinct mechanisms of platelet aggregation as a consequence of different shearing flow conditions. J Clin Invest 1998; 101: 479-86.

26. Savage B, Almus-Jacob F, Ruggeri ZM. Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. Cell 1998; 94: 657-66.

27. Kroll MH, Hellums JD, McIntire LV, Schafer AI, Moake JL. Platelets and shear stress. Blood 1996; 88: 1525-41.

28. Guyer DA, Moore KL, Lynam EB, Schammel CMG, Rogelj S, McEver RP, Sklar L. P-selectin glycoprotein ligand-1 (PSGL-1) is a ligand for L-selectin in neutrophil aggregation. Blood 1996; 2415-21.

29. Taylor AD, Neelamegham S, Hellums JD, Smith CW, Simon SI. Molecular dynamics of the transition from L-selectin- to beta 2-integrin-dependent neutrophil adhesion under defined hydrodynamic shear. Biophys J 1996; 71: 3488-500.

30. Neelameghan S, Taylor AD, Burns AR, Smith CW, Simon SI. Hydrodynamic shear shows distinct roles for LFA-1 and Mac-1 in neutrophil adhesion to intracellular adhesion molecule-1. Blood 1998; 92: 1626-38.

31. Konstantopoulos K, Neelamegham S, Burns AR, Hentzen E, Kansas GS, Snapp kR, Berg

EL, Hellems JD, Smith CW, McIntire LV, Simon SI. Venous levels of shear support neutrophil-platelet adhesion and neutrophil aggregation in blood via P-selectin and beta-2 integrin. Circulation 1998; 98: 873-82.

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

33. Liu L, Freedman J, Hornstein A, Fenton JW 2nd, Song Y, Ofusu FA. Binding of thrombin to the G-protein-linked receptor, and not to glycoprotein Ib, precedes thrombin-mediated activation. J Biol Chem 1997; 272: 1997-2004.

34. De Cristofaro R, Landolfi R. Thermodynamics of substrates and reversible inhibitors binding to the active site cleft of human alpha thrombin. J Molec Biol 1994; 239: 569-77.

35. Coller BS. A new murine monoclonal antibody reports an activation-dependent change in the conformation and/or microenvironment of the platelet glycoprotein IIb-IIIa complex. J Clin Invest 1985; 76: 101-8.

36. EPIC Investigators. Use of a monoclonal antibody directed against the platelet glycoprotein GPIIb/IIIa receptor in high-risk coronary angioplasty. N Engl J Med 1994; 330: 956-61.

37. Hartman GD, Egbertson MS, Halczenko W, Laswell WL, Duggan ME, Smith RL, Naylor AM. Non-peptide fibrinogen receptor antagonists. 1. Discovery and design of exosite inhibitors. J Med Chem 1992; 36: 4640-2.

38. RESTORE Investigators. Effect of platelet glycoprotein IIb/IIIa blockade with tirofiban on adverse cardiac events in patients with unstable angina or acute myocardial infection

undergoing coronary angioplasty. The RESTORE Investigators. Randomized efficacy study of tirofiban for outcomes and restenosis. Circulation 1997; 96: 1445-53.

39. Phillips DR, Scarborough RM. Clinical pharmacology of eptifibatide. Am J Cardiol 1997; 80: 11B-20B.

40. Schror K. Antiplatelet drugs. A comparative review. Drugs 1995; 50: 7-28.

41. Weitz JI. Low-molecular-weight-heparins. N Engl J Med 1997; 337: 688-98.

42. Weitz JI, Hudoba M, Massel D, Maraganore J, Hirsh J. Clot-bound thrombin is protected from inhibition by heparin-antithrombin III but is susceptible to inactivation by antithrombin III-independent inhibitors. J Clin Invest 1990; 86: 385-91.

43. Hauptmann J, Sturzebecher J. Synthetic inhibitors of thrombin and factor Xa: from bench to bedside. Thromb Res 1999; 93: 203-41.

44. Andre P, Hamaud P, Bal dit Solllier C, Drouet V, Garfinkel LT, Uzan A, Drouet Lo. Guinea pig blood: a model for the pharmacological modulation of the GPIb/IX-vWF axis. Thromb Res 1996; 83: 127-36.

45. Yamamoto H, Vreys I, Stassen JM, Yoshimoto R, Vermylen J, Hoylaerts MF. Antagonism of vWF inhibits both injury induced arterial and venous thrombosis in the hamster. Thromb Haemost 1998; 79: 202-10.

46. Bini A, Fenoglio JJ Jr, Mesa-Tejada R, Kudryk B, Kaplan KL. Identification and distribution of fibrinogen, fibrin and fibrin(ogen) degradation products in atherosclerosis. Arteriosclerosis 1989; 9: 109-21.

47. Kuijper PHM, Torres HIG, Lammers JWJ, Sixma JJ, Koenderman L, Zwaginga JJ. Platelet and fibrin deposition at the damaged vessel wall: cooperative substrates for

neutrophil adhesion under flow conditions. Blood 1997; 89: 166-75.

48. GUSTO Investigators: Global Use of Strategies to Open Occluded Arteries IIa Investigators. Randomized trial of intravenous heparin versus recombinant hirudin for acute coronary syndromes. Circulation 1994; 90: 1631-7.

49. Ginsberg MH, Plow EF. Fibronectin expression on the platelet surface occurs in concert with secretion. J Supramolec Structure 1981; 17: 91-8.

50. Goldsmith HL, McIntosh FA, Frojmovic MM. The kinetics of thrombin- and SFLLRN-induced aggregation of human platelets in flow through tubes. Biorheology 1998; 35: 53-68.

51. Sixma JJ, van Hemel BM, Galanakis DK, Rooney MR, Scharrer I, de Groot PG. Mutations in the gamma chain of fibrinogen between γ315 and γ322 are associated with an absence of platelet adhesion under conditions of flow. Blood 1998; 92(SuppI):Abstr # 1432.

52. Schielen WJG, Adams HPHM, van Leuven K, Voskuilen M, Tesser GJ, Nieuwenhuizen W. The sequence gamma-(312-324) is a fibrin specific epitope. Blood 1991; 77: 2169-73.

53. Rabhile-Sabile S, Thibert V, Legrand C. Thrombospondin peptides inhibit the secretion-dependent phase of platelet aggregation. Blood Coag and Fibrinolys 1996; 7: 237-40.

Appendix 1

Surface-Secreted von Willebrand Factor Mediates Aggregation of ADP-Activated Platelets at Moderate Shear Stress: Facilitated by GPIb but Controlled by GPIIb-IIIa

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1. Summary

We previously showed that ADP activation of washed human platelets in plasma-free suspensions supports aggregation at moderate shear stress (0.4-1.6 Nm^{-2}) in Poiseuille flow. Although most activated platelets expressed maximal fibrinogen-occupied GPIIb-IIIa receptors, aggregation appeared to be independent of bound fibrinogen, but blocked by the hexapeptide GRGDSP. Here, we tested the hypothesis that von Willebrand factor (vWF) secreted and expressed on activated platelets mediates aggregation at moderate shear rates from 300 to 1000 s^{-1} corresponding to shear stresses from 0.3 to 1.1 Nm^{-2}. Relatively unactivated platelets (<15% expressing prebound fibrinogen) were prepared from acidified citrated platelet rich plasma (cPRP) by single centrifugation with 50 nM stable prostacyclin derivative ZK 36374 and resuspended in Tyrodes-albumin at 5×10^4 cells μl^{-1}. Flow cytometric measurements with monoclonal antibody (mAb) 2.2.9 reporting on surface-bound vWF, and with mAb S12 reporting on α-granule secreted P-selectin, showed that 65% and 80%, respectively, of all platelets were maximally activated with respect to maximal secretion and surface expression of these proteins. "Resting" washed platelets exhibited both surface-bound vWF and significant P-selectin secretion. We showed that mAbs 6D1 and NMC4, respectively blocking the adhesive domains on the GPIb receptor recognizing vWF, and on the vWF molecule recognizing the GPIb receptor, partially inhibited ADP-induced aggregation under shear in Couette flow, the degree of inhibition increasing with increasing shear stress. In contrast, mAb 10E5, blocking the vWF binding domain on GPIIb-IIIa, essentially blocked all aggregation at the shear rates tested. We conclude that vWF, expressed on ADP-activated platelets, is at least the predominant cross-bridging molecule mediating aggregation at moderate shear stress. There is an absolute requirement for free activated GPIIb-IIIa receptors, postulated to interact with platelet-secreted, surface bound vWF. The GPIb-vWF cross-bridging reaction plays a facilitative role becoming increasingly important with increasing shear stress. Since aurin tricarboxylic acid, which blocks the GPIb binding domain on vWF, was also found to completely block aggregation in Poiseuille flow, we conclude that it too affects the GPIIb-IIIa interaction.

2. Introduction

In a previous paper, we reported that, in the absence of exogenous fibrinogen, ADP-induced activation of multiple-washed and centrifuged human platelets suspended in Tyrodes-albumin supported aggregation at moderate shear rates in Poiseuille flow (mean values, 335 and 1335 s^{-1}) corresponding to shear stresses of 0.4 and 1.6 Nm^{-2} (1). Although 54% of the resting and >75% of the ADP-activated, washed cells expressed maximal fibrinogen-occupied GPIIb-IIIa receptors (as monitored with FITC-labelled monoclonal antibody 9F9), the aggregation appeared to be independent of the bound fibrinogen, though blocked by the hexapeptide GRGDSP which did not alter the prebound fibrinogen. In addition, $F(ab')_2$ fragments of a polyclonal antibody to human fibrinogen which did not alter the extent of prebound fibrinogen, had almost no effect on the aggregation. Moreover, relatively unactivated washed platelets (14% of resting platelets expressing prebound fibrinogen), prepared by single centrifugation in an acid medium in the presence of the stable prostacyclin derivative, ZK 36374, and resuspension in Tyrodes-albumin, also supported ADP-induced aggregation at shear rates of 335 and 1335 s^{-1} in the absence of exogenous fibrinogen.

We therefore postulated that another adhesive protein, likely von Willebrand factor (vWF), secreted during platelet isolation and/or in flow at sufficiently high shear stress, mediates the observed aggregation following ADP-activation of the platelets. Even at the low shear rates (~100 s^{-1}) estimated to exist in stirred aggregometer vials, soluble vWF can mediate aggregation of ADP-activated platelets via binding to activated GPIIb-IIIa receptors in the absence of exogenous fibrinogen (2, 3). The aim of the present paper was to explore the postulated cross-bridging role of platelet surface-expressed vWF, by testing the single-washed, relatively unactivated platelets, before and after addition of agonist, for α-granule release and secretion and surface expression of vWF, and to compare the results with those of platelets in cPRP, in which freshly-prepared cells are known to be unactivated (4). In addition, we studied the effect on ADP-induced aggregation at moderate shear stress, of monoclonal antibodies (mAbs) LJ-152B/6 (152B/6) and NMC4, respectively blocking the GPIIb-IIIa and GPIb binding domains on vWF (5, 6), of mAbs 6D1 and 10E5, respectively blocking the vWF binding domains on GPIb (7) and GPIIb-IIIa (8), and of aurin tricarboxylic acid (ATA) reported to block the GPIb binding domain on vWF (9), using both a variable shear (Poiseuille flow) and a uni-

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form shear field (Couette flow; 4) at moderate shear stresses, from 0.3 to 1.1 Nm^{-2}.

Others have carried out experiments in free-flowing suspensions of washed platelets and blood subjected to high shear stress in Couette viscometers in the absence of extrinsic activators (5, 10-12). At shear stresses >8 Nm^{-2}, platelets do aggregate, but only in the presence of vWF, either added or released from the platelets. It is remarkable that, in our previous work, we found that, for the multiple-washed cells at 3×10^5 μl^{-1}, the critical shear stress for the onset of aggregation with 0.7 μM ADP was about 20-fold lower than that observed by Ikeda et al. (5). If vWF plays a major role in the ADP-induced aggregation at the much lower shear stress, one would expect a significantly increased secretion and surface expression of the adhesive protein on the ADP-activated washed platelets.

3. Materials and Methods

3.1. *Reagents*

Frozen aliquots of 20 mM ADP in Tyrodes and of 1.6 mM PMA in 100% ethanol (Sigma Chemical Co., St. Louis, MO) were warmed to room temperature before use and diluted in Ca^{++}-free Tyrodes-albumin solution. Electron microscope grade glutaraldehyde (J. B. EM Services, Pointe Claire, Quebec) was diluted to 0.5-0.8% (vol/vol) in Isoton II (Coulter Electronics Inc., Hialeah, FL). The stable prostacyclin derivative, ZK 36374 (13) was a generous gift of Dr. T. Krais, Schering Corporation, Berlin, Germany. Frozen aliquots of 10 μM ZK in saline containing 0.13% $NaHCO_3$ were warmed to room temperature before use and added to give the desired concentration.

Monoclonal antibodies 152B/6 (6) and NMC4 (5), which block the GPIIb-IIIa and GPIb domains on vWF, respectively, and mAb 2.2.9, specific to a vWF adhesion-independent domain with one site per 220 kDa repeating unit present at the COOH-terminal domain of vWF (14), were generously provided by Dr. Z. Ruggeri (Scripps Institute, La Jolla, CA), with NMC4 originally obtained from Dr. A. Yoshioka (Nara Medical University, Japan). Monoclonal antibodies 6D1, directed against the vWF binding site on GPIb (7) and 10E5, against the GPIIb-IIIa binding domain for soluble fibrinogen or vWF (8), were provided by Dr. Barry Coller (Mount Sinai Medical Center, New York, NY). Aurin tricarboxylic acid (2900 Da fraction), which blocks the GPIb binding domain on vWF and the associated ristocetin-induced platelet aggregation (15), was the generous gift of Dr. M. Weinstein (FDA, CBER, Rockville Pike, MD); mAb 9F9, specific for the receptor-induced binding site (RIBS) on fibrinogen bound to its activated GPIIb-IIIa receptor (16), was obtained from Dr. A. Budzynski (Temple University, Philadelphia, PA), and mAb S12, specific to platelet α-granule membrane protein GMP-140 (17), was a gift from Dr. R. McEver, Oklahoma Medical Research Foundation, Oklahoma City, OK. Labelling of antibodies with fluorescein isothiocyanate (FITC; Isomer I, on celite 10%, Boehringer Diagnostics, La Jolla, CA) was carried out as previously described (18).

3.2. *Preparation of Platelet-rich Plasma and Washed Platelet Suspensions*

Citrated PRP (cPRP) was prepared from venous blood collected from healthy human donors into 1/10 volume of 3.8% sodium citrate and processed as previously reported (1). Relatively unactivated washed platelets were prepared from cPRP by a single centrifuging and washing procedure as previously described (<15% expressing pre-bound fibrinogen; 1). Essentially, the procedure involved acidifying cPRP, centrifuging in the presence of 50 nM ZK 36374 and redispersing the platelet pellets in modified, Ca^{++}-free Tyrodes-albumin (136 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 1.0 mM $MgCl_2$, 5.6 mM glucose, 0.35% BSA). The platelet-rich Tyrodes (PRT) was then diluted with normal Tyrodes-albumin containing 2.0 mM $CaCl_2$ added just before activation and shear, to yield 5.0×10^4 cells/μl in the flow experiments. The fibrinogen concentration in solution was estimated to vary from 9 to 12 nM, an order of magnitude below the value reported for the fibrinogen-platelet receptor dissociation constant, K_d ~100-200 nM (19-21).

3.3. *Estimation of Activation of Washed and ADP-stimulated Platelets*

Twenty-five μl of washed platelets in Ca^{++}-free Tyrodes were mixed with 4.5 to 5.5 μl of FITC antibody and incubated for about 30 min. before quench-diluting the reaction with 10 vols of Ca^{++}-free Tyrodes, and analyzed in the flow cytometer within 30-60 s to minimize any post-dilution time-dependent changes (18). For ADP- or PMA-stimulated platelets, 22.5 μl of washed cells were rapidly mixed with 2.5 μl of activator (rapid hand swirling of the tube for ~1 s), 10 s before addition of the FITC antibody. The final concentrations of mAb 152B/6, 2.2.9 and S12 were 133, 173 and 100 nM, respectively. The presence of 1 mM $MgCl_2$, in the Ca^{++}-free Tyrodes normally allows "physiologic" studies of PAC1 and 9F9 binding to ADP-activated platelets, in the absence of confounding aggregation (1, 16-17). We nonetheless confirmed that identical equilibrium fluorescence histograms and platelet subpopulation distributions were obtained when directly comparing 9F9, S12 or 152B/6 binding to platelets activated with 5 μM ADP or 0.2 μM PMA in PRT with and without 2 mM $CaCl_2$ added 30 s before the activator.

The platelet suspensions were analyzed for 2500-5000 cells in a FACSCAN flow cytometer (Becton Dickinson Canada, Mississauga, Ontario) as previously described (18), with particular attention to the bimodal distribution of fluorescence due to resting (P^0) and maximally-activated platelets (P^*), as previously described (22). Thus, the fraction of cells activated are reported as $\%P^*$ for bimodal, subpopulation responses, or simply as % activated platelets for partially unimodal responses, where the entire platelet population undergoes a partial activation.

3.4. *Flow Devices*

Tube flow was used to evaluate the inhibition of shear-induced platelet aggregation by mAb LJ-152B/6 and ATA, reagents available in relatively large quantities, since a minimum of 10 ml of washed platelet suspension was required per run. All other inhibitors, mAbs NMC4, 6D1 and 10E5, were evaluated in the micro-Couette device which required only 400 μl of suspension per run.

3.4.1. *Poiseuille flow.* Platelet-rich Tyrode suspension and ADP solutions flowing from independent infusion pumps (Harvard Apparatus, Bedford, MA) were rapidly mixed in a stirred chamber of 50 μl volume at a fixed flow ratio, PRT:ADP = 9:1 (23). The suspensions flowed out into 0.76 mm i.d. polyethylene tubing (Clay Adams, Parsippany, NJ) from 2 cm to 10 m long at a volume flow rate, $Q = 108$ μl s^{-1}, corresponding to volume averaged mean tube shear rates $\langle G \rangle = 32Q/15\pi R_o^3 = 1335$ s^{-1}, R_o being the tube radius (23). The mean transit times (mean time of exposure to ADP), $\langle t \rangle = X/\langle U \rangle$, where X is the distance of flow along the tube and $\langle U \rangle$ is the mean linear fluid velocity, ranged from <0.1 to 42 s. Known volumes of suspension (~300 μl) were collected into 4.5 ml 0.5% isotonic glutaraldehyde, thereby arresting the reaction.

3.4.2. *Couette flow.* The platelet suspensions were sheared in an annulus of thickness h = 0.5 mm between concentric plexi-glass cylinders of a micro-Couette device (4). The outer cylinder, radius 5.5 mm, was stationary. By means of a high precision step motor, the inner cylinder, radius R = 5.0 mm, rotated at variable angular velocity, ω, corresponding to shear rates, G, given by $G = R\omega/h$ from 1 to 1000 s^{-1} ($h \ll R$). Adenosine diphosphate was briefly (<2 s) mixed with the platelet suspension using a disposable plastic pipette, and 400 μl transferred into the Couette after first lifting out the inner cylinder. The inner cylinder was lowered and the suspension sheared for a given time before arresting motion. A 30 μl subsample of suspension was then collected from a port on the outer cylinder situated just above the base of the inner cylinder, and immediately fixed in 7 vol of 0.8% glutaraldehyde, first discarding 20 μl of dead volume. The volume between the lower surface of the inner cylinder and the base of the outer cylinder is not sampled during these operations.

Data analysis – Poiseuille Flow. The number concentration and volume of single platelets and aggregates were measured using an electronic particle and

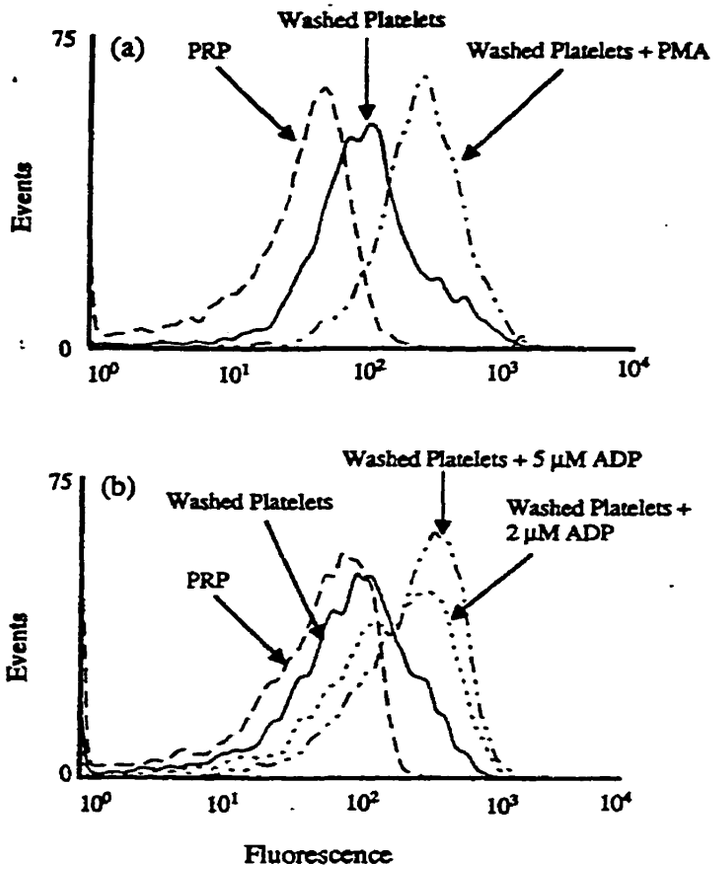

Fig. 1 Flow cytometric measurements of the extent of surface-bound vWF on washed platelets before and following activation with (a) 0.2 μM PMA and (b) 2 and 5 μM ADP, as monitored with mAb 2.2.9. The solid line histograms show mAb-binding to resting washed platelets, compared to platelets in cPRP (dashed line histogram). The dashed-dotted and dotted line histograms show binding to PMA- or ADP-driven maximally activated washed platelets. Data for the computed % activated cells in this and the following two figures are given in Table I

sizing system (Multisizer II, Coulter Electronics Inc., Hialeah, FL) to generate 250 class log-volume histograms over the equivalent sphere volume range 1-10^5 μm^3, as previously described (23). Computer integration of the log-volume histograms yielded the number concentration and volume fraction of particles between lower and upper volume limits. Platelet aggregation, PA, as defined by the fraction of single platelets incorporated into aggregates after a mean transit time t, PA = 1- N_t/N_o, was obtained from the measured number of single cells initially present, N_o, and those after time time <t>, N_t. Individual histograms from multiple donors were averaged, resulting in a histogram of the mean class volume fraction normalized to the maximum class content at <t> = 0 (24).

3.5. *Data analysis – Couette Flow*. The glutaraldehyde-fixed samples were analyzed in an electronic particle counter (Elzone 80xy, Particle Data Inc., Elmhurst, IL) to obtain the fractional decrease in the particle number concentration with time. Here, PA was defined in terms of the fractional decrease in number of *all* particles present (single cells and aggregates). Since the number of singlets is considerably greater than the number of aggregates over much of the time course of aggregation, PA defined in terms of the total number of particles is not significantly different from that defined in terms of the decrease of single cells. Alternatively, in some experiments, N_t/N_o was readily determined from an analysis of the flow times, T, needed to count a given number of particles (4,000) on the FACSCAN cytometer, as previously described for similar studies of neutrophil aggregation (25), where $N_t/N_o = T_o/T_t$. In other experiments, N_t/N_o was determined from measurements of the number of particles in platelet suspensions diluted a further 7 times after 20 s of flow (fixed volume).

Capture efficiencies, α_G, representing the fraction of the total number of two-body collisions between platelets resulting in doublet formation, were computed from the initial slopes, $dPA/dt|_{t=o}$, of the measured PA-time curves (representing the collision capture frequency) assuming the two-body collision frequency, FG per unit volume of suspension, is given by (26):

$$F_G = \frac{16}{3} G b^3 N_0^2$$

where G is the shear rate, b the radius of the equivalent platelet sphere (~1.13 μm; 27), N_o is the number concentration of platelets before the onset of shear, and hence:

$$\alpha_G = dPA/dt|_{t=o}/F_G$$

Wherever possible, data are presented as mean values ± one standard error of the mean (SEM).

4. Results

4.1. *Flow Cytometric Determinations of Fibrinogen and vWF on Washed Platelets*

4.1.1. *Surface-bound vWF*. In our previous studies of shear-induced aggregation of single centrifuged and washed platelets with 2 or 5 μM ADP in the absence of added plasma proteins, the measured aggregation appeared to be independent of platelet surface fibrinogen, and we hypothesized that aggregation was mediated by platelet secretion and surface-expressed vWF (1). We therefore compared the surface expression of the number of vWF molecules on resting platelets in cPRP, with those on washed platelets before and after activation with PMA (expected to yield maximal α-granule secretion and surface expression of vWF; 17, 28), shown in Fig. 1a, as well as with washed platelets before and after activation with 2 and 5 μM ADP, shown in Fig. 1b. The FITC-labelled mAb 2.2.9 was used to report on the surface-bound vWF, as it reacts with the COOH terminus of the vWF repeating 220 kDa subunits, with no known inhibitory activity on vWF function (14).

Figure 1 clearly shows that, both before and after activation with ADP, washed platelets exhibit two populations consisting of relatively unactivated platelets, and of platelets maximally expressing vWF (P*), as compared to one population when they are fully activated with 0.2 μM PMA. The % platelets expressing maximal mAb binding, corresponding to the subpopulation P*, is shown in Table I. One of the three donors' platelets evaluated with mAb 2.2.9 showed a unimodal activation of all cells. In all cases, however, there was unequivocal evidence for surface expression of vWF, with a significant increase in %P* upon activation with ADP (Table I).

Table I Surface-bound fibrinogen and vWF on washed platelets, before and after "physiologic" activation

Activator Added	Mean % Activated Platelets ± SEM [a]			
	9F9 (Fibrinogen)	2.2.9 (vWF)	152B/6 (vWF*) [c]	S12 (P-selectin)
None	17 ± 6 (5)	43 ± 9 (1/3) [b]	0 (2)	56 ± 8 (3/9)
2 - 5 μM ADP [d]	44 ± 15 (4)	65 (2)	63 (2/2)	81 ± 9 (6)
0.2 μM PMA	98 ± 2 (3)	100 (1)	100 (2)	98 ± 2 (5)

[a] % P* subpopulation expressing maximal mAb binding, unless otherwise indicated.

[b] Number of preparations showing unimodal shift/total number of samples.

[c] vWF* corresponds to adhesive domain for activated GPIIb-IIIa.

[d] 2 μM ADP for 9F9 and 2.2.9; 2, and 2 - 5 μM ADP for S12 with identical results for this range of [ADP]; 5 μM ADP for 152B/6, the same conditions as used in the aggregation studies.

4.1.2. *α-Granule secretion.* As illustrated in Fig. 2, the α-granule secretion marker, P-selectin, probed with mAb S12, also showed that major secretion had occurred in the subpopulation P* of the resting washed platelets. When caused to maximally secrete with 0.2 μM PMA, the washed platelets expressed the same number of bound S12 molecules as did similarly activated platelets in cPRP. The resting washed platelets of two-thirds of nine donors showed the presence of a subpopulation, the remainder exhibiting unimodal partial activation of the entire platelet population (Table 1). However, addition of 2-5 μM ADP resulted in the identical significant increase in %P* (maximal secretion) irrespective of unimodal or subpopulation behaviour, i.e. a similar majority of platelets became maximally activated (Table 1).

4.1.3. *Surface-bound fibrinogen.* As previously reported (1), Fig. 3 shows that only a minor fraction of the resting washed platelets expressed surface-bound fibrinogen (reported by mAb 9F9), as compared to 100% P* with fibrinogen surface expression seen with PMA activation. However, addition of 2 μM ADP, increased the % P* containing surface fibrinogen to 44%, significantly lower than the P-selectin and vWF secretion and surface expression on ADP-activated washed platelets (Table 1).

4.1.4. *GPIIb-IIIa binding domain of vWF.* About a third to a half of all platelets had maximally expressed surface-bound vWF, as reported by mAb 2.2.9, on the washed platelets prior to the addition of any extrinsic activator. It was therefore surprising that there was no significant binding of mAb 152B/6 to the adhesive domain on vWF for activated GPIIb-IIIa (vWF*; Table 1), with fluorescence values equivalent to those of resting platelets in cPRP (data not shown). While addition of 5 μM ADP only changed the %P* expressing maximal vWF from ~43 to 65% (as reported by mAb 2.2.9), this ADP activation caused all of the platelets to express 63% of the 152B/6 maximal binding sites seen on platelets maximally activated with PMA (Table 1). In fact, this was very similar to the 72% activation of all platelets in cPRP activated with 5 μM ADP (n = 2), compared to PMA activation for the same two donors (data not shown). Lower concentrations of ADP (0.7-1.0 μM), insufficient to promote shear-induced aggregation in suspensions of 5×10^4 platelets μl^{-1} (1), were also unable to yield any significant binding of 152B/6 to washed platelets, in contrast to measurable binding observed with similarly activated platelets in cPRP (data not shown). It is therefore expected that mAb 152B6 will only begin to bind to washed platelets following ADP activation, and may therefore not be an effective inhibitor.

4.2. *Effects of Antibodies and ATA on Platelet Aggregation*

4.2.1. *Reagent concentrations and specificity.* To determine the concentration of ATA and of mAb NMC4, directed against the GPIb-binding domain on vWF, required to completely inhibit soluble vWF-mediated platelet aggregation, washed platelet suspensions containing 10% platelet-poor plasma were stirred with 0.3-1.5 mg/ml ristocetin at 1000 rpm in an aggregometer for 2 min. The suspensions were then rapidly fixed with 0.8% glutaraldehyde and a particle size analysis carried out. In the case of ATA, aggregation was totally inhibited at concentrations >30 μM. In the case of NMC4 concentrations greater than 100 nM were required to completely inhibit the reaction, but we chose 80 nM as used by Ikeda et al. (12) to block high shear stress-induced aggregation. In order to optimize efficient use of mAb 152B/6, we tested the antibody at 31 and 310 nM, representing a 4.3× lower, and 2.3× higher concentration than that (133 nM) shown by Ikeda et al. (12) to block high shear stress-induced aggregation.

In the stirred vials of an aggregometer, ≤10 μg/ml of the mAbs 6D1 and 10E5 have been shown to block aggregation dependent on soluble vWF binding to GPIb (5) or GPIIb-IIIa (6) respectively, on platelets. These antibodies have also been shown to block high shear stress induced aggregation (≥6 N m^{-2}) of platelets in Couette flow (12). Using the micro-Couette, we confirmed the fact that, at a shear rate of 1000 s^{-1}, 10 μg/ml of mAb 6D1 could indeed block ristocetin-induced aggregation of washed platelets containing 21 nM soluble vWF monomer equivalents.

All of the mAb's tested were IgG1 types, with mAB 152B/6 effectively serving as the ideal control, as it had no measurable effect on platelet aggregation at the shear rates tested, while binding both non-specifically as well specifically to vWF present on the ADP-stimulated platelets.

4.2.2. *Aggregation in Couette flow.* We previously reported that, in the absence of extrinsically-added fibrinogen, single-centrifuged and washed platelet supensions containing 5×10^4 cells/μl aggregated with 5 μM ADP in Poiseuille flow at mean tube shear rates <G> = 335 and 1335 s^{-1}, the rate and extent of aggregation increasing with increasing

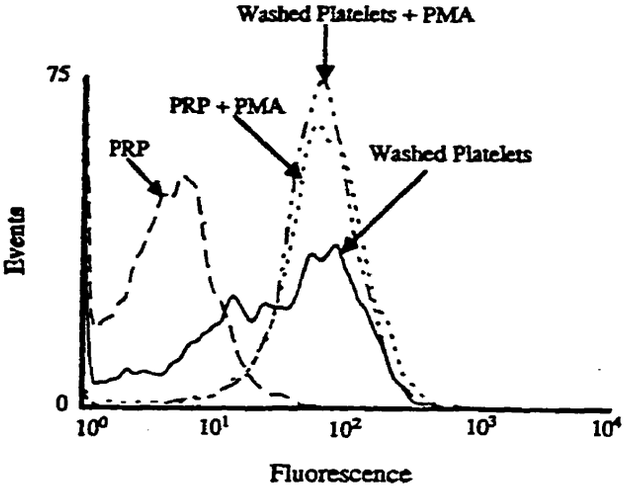

Fig. 2 Histograms, as in Fig. 1, of the extent of surface-bound, α-granule secreted P-selectin, before (solid line) and following (dashed-dotted line) activation by 0.2 μM PMA, as monitored by mAb S12. Washed platelets are compared with those in resting (dashed line) and PMA-activated (dotted line) cPRP

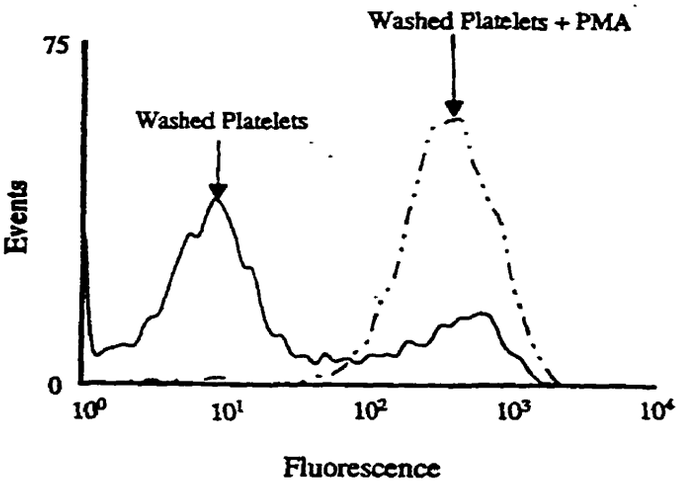

Fig. 3 Histograms, as in Fig. 1, of the extent of surface-bound fibrinogen before (solid line) and following (dashed-dotted line) activation with 0.2 μM PMA, as monitored by mAb 9F9

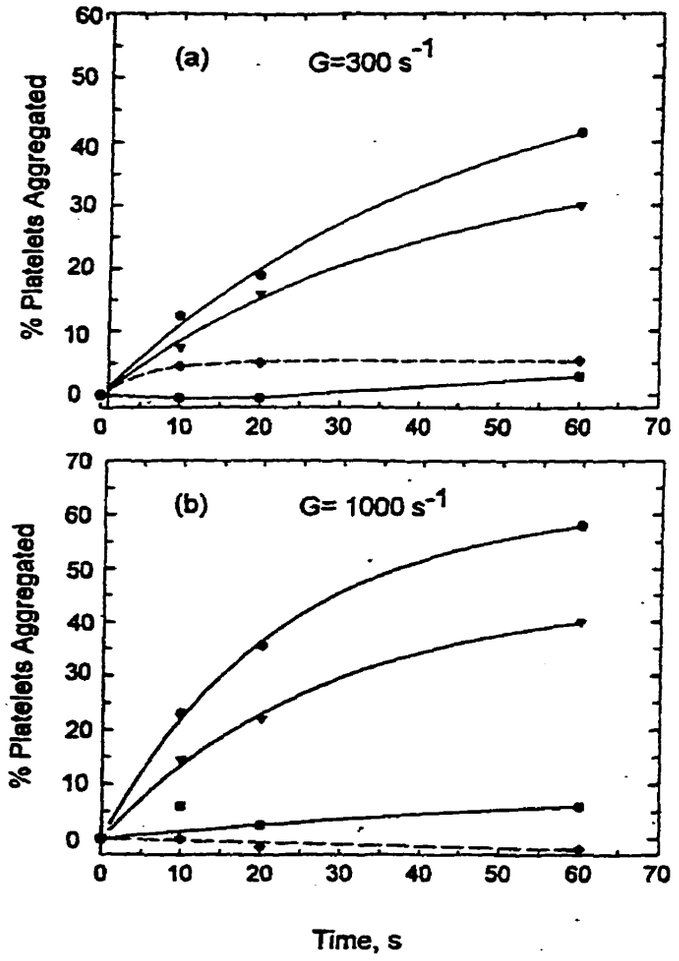

Fig. 4 Inhibition of aggregation of washed platelets, induced by 5 μM ADP, in Couette flow at G = 300 s^{-1} (a) and 1000 s^{-1} (b), shown for a single experiment. Plot of the time course of % platelet aggregation over 1 min in the absence of antibody (●), and in the presence of 10 μg/ml of mAbs 6D1 (▼) and 10E5 (■). The dashed line (◆) is the control run in the absence of ADP and antibody

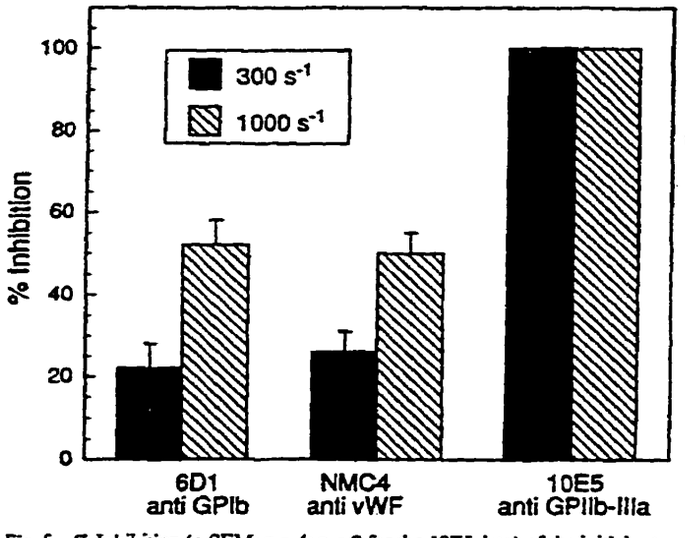

Fig. 5 % Inhibition (+ SEM; n = 4; n = 2 for the 10E5 data) of the initial rates of platelet aggregation by antibodies targeted against vWF or its receptors, GPIb and GPIIb-IIIa. The suspensions were sheared at G = 300 s^{-1} (except for NMC4, at G = 335 s^{-1}) and 1000 s^{-1}. Kinetics of the aggregation are shown in Figs. 4 and 6

<G>. The experiments were repeated in the micro-Couette at uniform shear rates of 300 and 1000 s^{-1} with the aim of obtaining values of the initial rates of aggregation (approximating initial rates of doublet formation) and thus to derive values of the collision capture efficiencies, α_G. The inhibitory effects of select mAbs could then be determined. We had previously shown (unpublished results) that, in the absence of inhibitor, the time course of aggregation at <G> = 335 and 1335 s^{-1} was very similar to that observed in Poiseuille flow, the % aggregation at given times being within ±5% of that found in the tube. Plots of the time course of % platelet aggregation (%PA) induced by 5 μM ADP in the presence and absence of inhibitors are shown in Figs. 4a and 4b. We found that for a >3-fold increase in G, from 300 to 1000 s^{-1}, α_G decreased by only 20% from 0.090 ± 0.005 (n = 4) to 0.070 ± 0.004 (n = 5). This result is in marked contrast to the 73% decrease in α_G over the same range of G, which was found in fibrinogen-driven aggregation of cPRP in the micro-Couette (4).

4.2.3 *Antibodies 6D1 and 10E5.* These antibodies were used to evaluate the roles of the vWF binding domains on GPIb and GPIIb-IIIa in mediating aggregation of activated platelets. Washed suspensions of 5×10^4 cells were preincubated with 10 μg/ml of the antibody for 5 min, and then immediately sheared in the device following addition of 5 μM ADP. The time course of aggregation over one minute, plotted in Figs. 4a and 4b at G = 300 and 1000 s^{-1}, respectively, clearly indicates shear-dependent inhibition of both the initial rate and extent of aggregation in the case of mAb 6D1, compared to almost total blockage of aggregation at both shear rates in the case of mAb 10E5. Thus, as shown in the histogram of Fig. 5, the degree of inhibition of the initial rate of aggregation (and therefore in α_G) due to mAb 6D1 increased from 22 ± 6% at G = 300 s^{-1} to 52 ± 6% at G = 1000 s^{-1}. Similarly, the decrease in the extent of aggregation after one minute of shear caused by mAbs 6D1 and 10E5, respectively, increased from 28 ± 1% and 87 ± 4% at G = 300 s^{-1} to 59 ± 5.5% and 89 ± 1% at G = 1000 s^{-1}.

4.2.4 *Antibody NMC4.* Given the partial inhibition of aggregation by mAb 6D1, we compared the effect of the complementary mAb NMC4, which binds to the GPIb adhesive domain on the vWF molecule (5). As shown in Fig. 6, washed platelets preincubated with the antibody for 5 min prior to addition of 5 μM ADP and sheared at G = 335 and 1000 s^{-1}, exhibited aggregation profiles similar to those of platelets preincubated with mAb 6D1. Thus, the initial rates of aggregation decreased by 26 ± 5 and 50 ± 3% (n = 4) at G = 335 and 1000 s^{-1}, respectively, corresponding to the values of % inhibition shown in Fig. 5.

4.3 *Aggregation in Poiseuille Flow*

4.3.1 *Antibody LJ-152B/6.* Although we found that mAb 152B/6, reported to bind to the GPIIb-IIIa domain of vWF (6), only bound to a subpopulation of these molecules after activation with 5 μM ADP (Table 1), we still tested its inhibitory effect on shear-induced aggregation. In order to enhance binding after activation with ADP, the washed platelets were incubated for 30 min with the antibody prior to shearing in the presence of 5 μM ADP at <G> = 1335 s^{-1}. We found that preincubation of the cells for 30 min with 31 or 310 nM 152B/6 had no significant effect on the rate or extent of aggregation. Thus, at <t> = 42 s, the extent of aggregation obtained in the presence of 310 nM antibody (52.2%) was not significantly different from that in the absence of antibody (49.3%; mean of two experiments).

4.3.2 *Aurin tricarboxylic acid.* In our laboratory (as described above), and by others (9), ATA has been shown to block the GPIb domain of vWF. Given that ATA is being evaluated as an antithrombotic agent in animal models (29, 30), and that ATA may affect both GPIb and GPIIb-IIIa

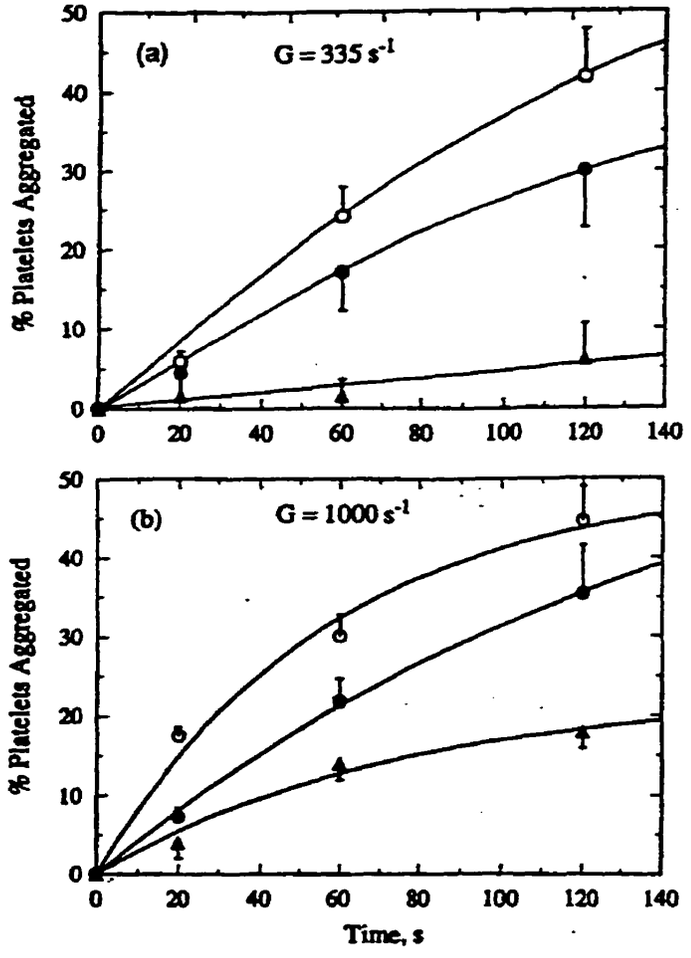

Fig. 6 Effect of monoclonal antibody NMC4 on aggregation in Couette flow induced by 5 μM ADP in washed platelets, 5 × 10^4 cells μl^{-1}, at (a) G = 335 s^{-1} and (b) 1000 s^{-1}. Plot of the time course of the mean % platelets aggregated (± SEM; n = 4) as a function of the time of shearing: ○ – in the absence of NMC4; ● – in the presence of 80 nM NMC4; ▲ – control run in the absence of ADP and NMC-4

interactions with their ligands (29), we wished to compare the inhibitory effects of ATA on platelet aggregation, with the similar but partial inhibition observed with the mAbs 6D1 and NMC4 directed exclusively at the GPIb and vWF domains required for cross-bridging of platelets.

By contrast, as illustrated in Figs. 7a and 7b, preincubation of the cells with 31 μM ATA completely abolished aggregation with 5 μM ADP in Poiseuille flow at <G> = 1335 s^{-1}. The plot of the fraction of single cells aggregated against <t> (Fig. 7a) shows that 104.4 ± 2.8% (S.E.M., n = 5) of single platelets remained unaggregated at <t> = 42 s, a result not significantly different from that, 99.5 ± 3.2% (n = 5), obtained in the control run, in which Tyrodes-albumin was infused instead of ADP, and no ATA was added. In the absence of ATA, however, there was significant ADP-induced aggregation: 24.7 ± 3.9% (n = 5) of single cells were incorporated into aggregates (Fig. 7a). The extent of aggregate growth at <t> = 42 s in the presence or absence of antibody is shown in Fig. 7b in the continuous volume fraction histograms in which the mean, normalized volume fraction of particles in each of the 250 classes is plotted against particle volume, over the range 1-10^5 μm^3. It is evident that, in the absence of ATA, a marked reduction in the height of the singlet peak resulted in the appearance of two bands of aggregates, between 30 and 200 μm^3, and between 800 and 10^4 μm^3.

5. Discussion

The results of the work reported above lend strength to the hypothesis that von Willebrand factor, secreted during the isolation and activation of the single centrifuged and washed platelets, and surface-bound on the activated cells, is required for ADP-induced aggregation at moderate shear stress in the absence of exogenously added fibrinogen, with a supporting (facilitating) role for GPIb and an absolute (regulating) role for GPIIb-IIIa.

(i) von Willebrand factor is indeed maximally expressed on 43% of platelets isolated in the PGI_2-mimetic (ZK) protected environment. We know from previous work (1) that such platelets do not aggregate when stirred in an aggregometer, or at low shear rate (42 s^{-1}) in tube or Couette flow, unless both fresh extrinsic fibrinogen and "physiologic" activation by ADP are provided.

(ii) Mutually adhesive domains on GPIb and vWF, blocked by mAbs 6D1 and NMC4, respectively, are only partially responsible for platelet aggregation at G ≥300 s^{-1}. These sites are predicted to facilitate maximal capture of platelets by the activated GPIIb-IIIa* interactions with platelet-secreted, surface-bound vWF. The im-

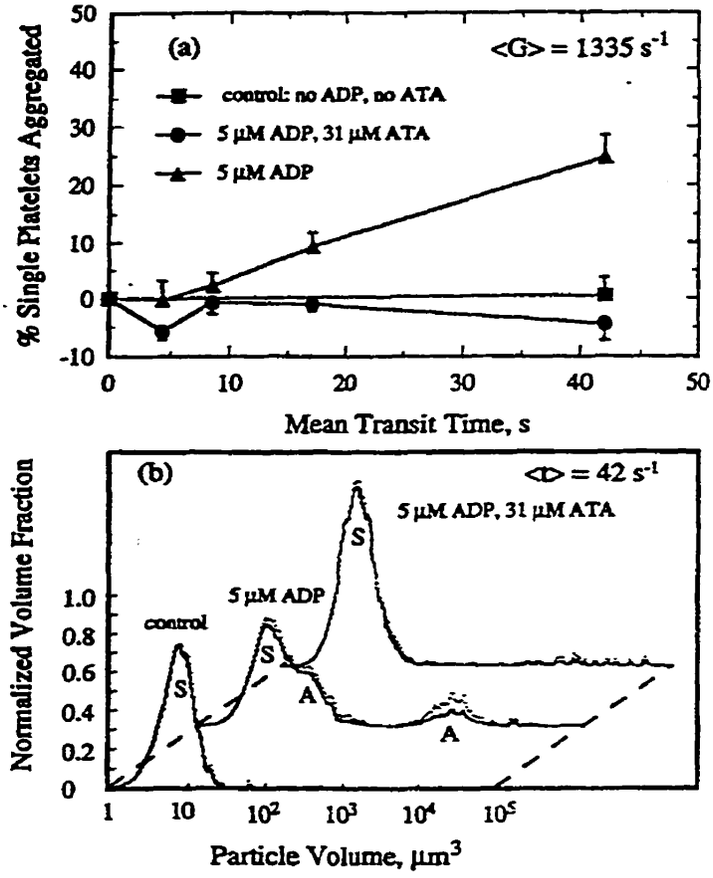

Fig. 7 Effect of 31 μM aurin tricarboxylic acid (ATA) on aggregation induced by 5 μM ADP in washed platelets, 5 × 10^4 cells μl^{-1}, undergoing Poiseuille flow at <G> = 1335 s^{-1}. (a) Plot of mean values of the percentage of single platelets aggregated (± S.E.M.; n = 5) as a function of mean transit time. The control run was carried out in the absence of ATA by infusing Tyrodes-albumin instead of ADP. (b) Comparison of aggregate growth in the presence and absence of ATA, and in the control run. Volume fraction histograms showing three-dimensional plots of the mean, normalized class volume fraction for the same data as in (a) vs particle volume at mean transit time = 42 s. S = singlets, A = aggregates; the dots represent S.E.M.

portance of the facilitative role for the GPIb-vWF cross-bridging reaction increases with increasing shear rate, as evidenced by the greater inhibition of PA at G = 1000 than at 300 s^{-1} (Fig. 5).

(iii) There is an absolute requirement for free activated GPIIb-IIIa receptors for crossbridging and capture of platelets at G = 300, 1000 or 1335 s^{-1}, as seen by the total blocking of aggregation by GRGDSP (1) or by 10E5 (Fig. 5).

(iv) ATA can completely block aggregation at <G> = 1335 s^{-1} in Poiseuille flow, under conditions demonstrated to block the GPIb/vWF/ristocetin-mediated aggregation, but predicted to also affect the GPIIb-IIIa interaction, absolutely required for optimal aggregation.

5.1 *Shear Rate-dependent Role of vWF*

The initial collision capture efficiencies, α_G, in the experiments with washed platelets activated by 5 μM ADP, shown in Figs. 4a and 4b, were 0.09 ± 0.01 (n = 4) and 0.07 ± 0.01 (n = 5) at G = 300 and 1000 s^{-1}, respectively. In a previous study of fibrinogen-driven ADP-induced aggregation in Couette flow, we found the relationship $\alpha_G = 53G^{-1}$, corresponding to $\alpha_G = 0.18$ and 0.05 at G = 300 and 1000 s^{-1}, respectively. Thus, α_G for *surface-secreted vWF-mediated* aggregation at G = 300 s^{-1} is already 50% of the maximal value for fibrinogen-driven aggregation, and at G = 1000 s^{-1} is even 40% more efficient. These results are consistent with an increasingly important role for vWF in mediating aggregation at higher shear rates (5, 10-12). Similarly, the inhibition of initial rates of vWF-driven aggregation of washed platelets via the vWF domain of GPIb on platelets (targeted by mAb 6D1), or by the GPIb domain on surface-bound vWF (targeted by mAb NMC4) was only 22% at G = 300 s^{-1}, but increased to 52% at G = 1000 s^{-1} (Fig. 5).

5.2 *Relative Roles of GPIb and GPIIb-IIIa*

At shear rates <100 s^{-1}, in stirred aggregometer vials, and in tube flow (1), the addition of fibrinogen is an absolute requirement for ADP-induced aggregation in washed platelet suspensions. Thus, the surface expressed vWF resulting from washing and centrifugation of the platelets, appears to be unable to mediate aggregation. However, above a critical threshold shear rate ~300 s^{-1}, the platelets will aggregate. At this moderate shear rate, it seems that the interaction between vWF and GPIb can largely be bypassed, since, as pointed out above, inhibition of the initial rates of aggregation by mAbs 6D1 and NMC4 is only minor. With increasing shear rate, however, the role of GPIb becomes increasingly important, as evidenced by the increased inhibition of the initial rate of aggregation.

We therefore propose a model for the shear dependent involvement of vWF in the aggregation of platelets in the absence of exogenously added fibrinogen, based on differences in the strength and lifetime of the bonds between the respective binding domains of vWF for the GPIb and GPIIb-IIIa receptors. First, it should be pointed out that much of the vWF secreted from the α-granules is likely associated with, and anchored to the GPIIb-IIIa receptors. Thus, it has been reported that freshly secreted vWF from thrombin-activated platelets cannot be prevented from binding to the cell surface using mAb 6D1 directed against the vWF binding site on GPIb, and is only ~72% inhibited from binding to GPIIb-IIIa by mAb 10E5 (31). Thus, it appears that vWF is partly secreted in association with GPIIb-IIIa, and mAb 10E5 is unable to inhibit this subpopulation of vWF from being released and surface bound to its receptors. Upon activation with ADP, it is proposed that the first reaction between the surface expressed and bound vWF with a GPIb receptor on an adjacent platelet results in the formation of rapidly reversible bonds having fast on and off rates, but which are relatively resistant to rupture by externally applied shear stress. The second reaction between an altered domain on vWF and a GPIIb-IIIa receptor on an adjacent platelet, results in the formation of a strong bond resistant to rupture at high shear stress, but with relatively slow on and off rates. Thus, with increasing shear rate, the first reaction becomes increasingly important in order to facilitate bond formation in the second reaction, and thus provide efficient capture of platelets, as seen at G = 1000 s^{-1}. There is, therefore, an obligatory role for GPIIb-IIIa at all shear rates, and a facilitative role for GPIb especially at high shear rates.

There are, in fact, good analogues for such a model, as in:

(i) the rapid reversible bond formation of L-selectin on neutrophils to counter receptors on endothelial cells enabling the neutrophils to roll along the vessel wall. In turn, this facilitates capture of the leukocyte via the firm bond formed between the integrin activated Mac-1 on the neutrophil, and its counter receptor on activated endothelial cells, required at modest shear rates (≤300 s^{-1}; 32).

(ii) the translocation of resting platelets via rapidly reversible bond formation between GPIb on resting platelets and vWF immobilized on a plastic surface. Again, this process facilitates the subsequent firm arrest of the platelets through activated GPIIb-IIIa (33).

It should be noted that, whereas the GPIb-vWF facilitated aggregation in the free flowing suspensions is largely bypassed at 300 s^{-1} via interactions with activated GPIIb-IIIa, this is not the case in the above quoted surface interactions of cells. There, rolling (facilitation of wall adhesion) is an absolute prerequisite for firm capture. This difference likely arises from the presence of activated GPIIb-IIIa on the ADP-stimulated platelets prior to the onset of vWF-GPIb cross-bridging reactions. It is, however, in marked contrast to high shear stress-induced platelet aggregation, in which the initial soluble vWF binding to GPIb leads to intracellular signalling and activation of GPIIb-IIIa (12), or to a similar observation for the translocation of resting platelets on surface-immobilized vWF with firm capture requiring subsequent platelet activation (33).

5.3 *Inabilty of mAb 152/6 to Block Aggregation*

Ikeda et al. (5) found that high shear stress-induced aggregation in hirudinized PRP in the presence of soluble vWF was completely blocked by 133 nM 152B/6, preincubated for only 5 min before shearing. By contrast, we found that, at moderate shear stress, there was no significant inhibition of the aggregation of the ADP-activated washed platelets even at 310 nM mAb when preincubated for 30 min prior to addition of agonist. The inability of this antibody to bind to surface-secreted vWF, maximally expressed on 43 ± 9% of all washed platelets prior to addition of extrinsic activator, as reported by mAb 2.2.9 (Table 1), suggests that the GPIIb-IIIa binding epitope is not accessible. However, following activation by 5 μM ADP, all of the platelets express about 63% of the maximal number of such epitopes suggesting that (i) the original vWF on "resting platelets" can be conformationally transformed, and/or (ii) newly-secreted vWF expresses these epitopes. Given the obligatory requirement for GPIIb-IIIa to mediate aggregation at 300 and 1000 s^{-1}, it appears that 152B/6 cannot access its GPIIb-IIIa domain on vWF in sufficient quantity to block aggregation, but that these ADP-dependent domains or other domains still not characterized are indeed required. Although 152B/6 has recently been shown to block soluble vWF binding to GPIIb-IIIa on thrombin-activated platelets

(14), it may not be an appropriate antibody for blocking the epitope on surface-bound vWF required for the actual cross-bridging reactions in platelet aggregation, or the rapid and perhaps irreversible binding required to block the shear-induced aggregation immediately following ADP addition may not occur with this antibody. Alternatively, a ligand other than vWF may bind to GPIIb-IIIa.

5.4 *The Role of ATA in Blocking Aggregation*

The striking difference between the total inhibition of the ADP-induced aggregation by ATA, and the partial, shear stress dependent inhibition by antibodies 6D1 and NMC4, the latter directed exclusively at the GPIb and vWF domains required for cross-bridging, suggests that ATA is also able to inhibit vWF binding to GPIIb-IIIa.

In this connection it is of interest to note that a somewhat related study concurrent with ours (34) has shown that cPRP pretreated with a low concentration of epinephrine (250 nM) can synergize aggregation induced by high shear stress (6 Nm^{-2}). Such aggregation is only partially inhibited by 6D1 or ATA, the latter in marked contrast to the total inhibition in our experiments. It should be noted, however, that Wagner et al. (34) used a less purified form of ATA (from Sigma Chemicals). Nevertheless their studies provide another example of the partial bypass of the GPIb-vWF interactions possible in high shear stress-induced aggregation when GPIIb-IIIa* is provided by a separate physiologic activation.

6. Concluding Remarks

We have shown that vWF secreted and surface expressed on ADP-activated, washed platelets can mediate aggregation at moderate shear stresses (0.3-1.1 Nm^{-2}), in contrast to the much higher shear stresses (>8 Nm^{-2}) required for aggregation of resting platelets with soluble plasma vWF (5). A useful analogue of the facilitating role of the GPIb-vWF crossbridging of platelets can be found in the models reported for neutrophil rolling on endothelium (32) and the translocation of platelets on vWF immobilized on a plastic surface (33).

With increasing shear rate, the vWF adhesive domain on GPIb apparently becomes increasingly essential for aggregation. The GPIIb-IIIa* domain on secreted and surface-bound vWF is also likely involved in aggregation, but cannot be blocked by mAb 152B/6 which can block soluble vWF binding to GPIIb-IIIa*. Thus, GPIb-vWF interactions facilitate and are increasingly important at moderate and high shear rates, to permit effective capture of platelets. We postulate that these most likely occur via GPIIb-IIIa-vWF cross-bridging between cells. The potential contributions of other surface-expressed proteins, especially P-selectin and thrombospondin to aggregation at moderate shear rate, are being explored.

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8. References

1. Goldsmith HL, Frojmovic MM, Braovac S, McIntosh F, Wong, T. Adenosine diphosphate-induced aggregation of human platelets in flow through tubes: III. Shear and extrinsic fibrinogen-dependent effects. Thromb Haemost 1994; 71: 78-90.
2. Timmons S, Hawiger J. von Willebrand factor can substitute for plasma fibrinogen in ADP-induced platelet aggregation. Trans Assoc Am Phys 1986; 99: 226-35.
3. De Marco L, Girolami A, Zimmermann TS, Ruggeri, Z. von Willebrand factor interaction with the glycoprotein IIb/IIIa complex; its role in platelet function as demonstrated in patients with congenital afibrinogenemia. J Clin Invest 1986; 77: 1272-7.
4. Xia Z, Frojmovic MM. Aggregation efficiency of activated normal or fixed platelets in a simple shear field: Effect of shear and fibrinogen occupancy. Biophys J 1994; 66: 2190-201.
5. Ikeda Y, Handa M, Kawano K, Kamata T, Murata M, Araki Y, Anbo H, Kawai Y, Watanabe K, Itagaki, I, Sakai K, Ruggeri Z. The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. J Clin Invest 1991; 87: 1234-40.
6. Berliner S, Niiya K, Roberts JR, Houghton RA, Ruggeri Z. Generation and characterization of peptide-specific antibodies that inhibit von Willebrand factor binding to glycoprotein IIb-IIIa without interacting with other adhesive molecules. Selectivity is conferred by Pro1743 and other amino acid residues adjacent to the sequence Arg1744-Gly1745-Asp1746. J Biol Chem 1988; 263: 7500-5.
7. Coller, BS, Peerschke EI, Scudder LE, Sullivan CA. Studies with a murine monoclonal antibody that abolishes ristocetin-induced binding of von Willebrand factor to platelets: additional evidence in support of GPIb as a platelet receptor for von Willebrand factor. Blood 1983; 61: 99-110.
8. Coller, BS, Peerschke EI, Scudder LE, Sullivan CA. A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins IIb and/or IIIa. J Clin Invest 1983; 72: 325-38.
9. Girma J-P, Fressinaud E, Christophe O, Roualt C, Obert B, Takahashi Y, Meyer D. Aurin tricarboxylic acid inhibits platelet adhesion to collagen by binding to the 509-695 disulphide loop of von Willebrand factor and competing with glycoprotein Ib. Thromb Haemost 1992; 68: 707-13.
10. Moake JL, Turner NA, Stathopoulos NA, Nolasco LH, Hellums JD. Involvement of large plasma von Willebrand factor (vWF) multimers and unusually large vWF forms derived from endothelial cells in shear stress-induced platelet aggregation. J Clin Invest 1986; 78: 1456-61.
11. Moake JL, Turner NA, Stathopoulos NA, Nolasco LH, Hellums JD. Shear-induced platelet aggregation can be mediated by vWF released from platelets, as well as by exogenous large or unusually large vWF multimers, requires adenosine diphosphate and is resistant to aspirin. Blood 1988; 71: 1366-74.
12. 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 GPIb in the initiation of shear-induced platelet aggregation. Thromb Haemost 1993; 69: 496-502.
13. Schor K, Darius H, Matzky R, Ohlendort R. The antiplatelet and cardiovascular actions of a new carbacyclic derivative (ZK 36374) – equipotent to PGI_2 in vitro. Arch Pharm 1981; 316: 252-5.
14. Goto S, Salomon DR, Ikeda Y, Ruggeri ZM. Characterization of the unique mechanism mediating the shear-dependent binding of soluble von Willebrand factor to platelets. J Biol Chem 1995; 270: 23352-61.
15. Weinstein M, Vosburgh E, Phillips M, Turner N, Chute-Rose L, Moake J. Isolation from commercial aurintricarboxylic acid of the most effective polymeric inhibitors of von Willebrand factor interaction with platelet glycoprotein Ib. Comparison with other polyanionic and polyaromatic polymers. Blood 1991; 78: 2291-8.
16. Shattil, SJ, Budzynski A, Scrutton MC. Epinephrine induces platelet fibrinogen receptor expression, fibrinogen binding, and aggregation in

whole blood in the absence of other excitatory agonists. Blood 1989; 73: 150-8.
17. Shattil SJ, Cunningham M, Hoxie JA. Detection of activation platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. Blood 1987; 70: 307-15.
18. Frojmovic MM, Wong T, van de Ven, T. Dynamic measurements of the platelet membrane glycoprotein IIb-IIIa receptor for fibrinogen by flow cytometry. I. Methodology, theory and results for two distinct activators. Biophys J 1991; 59: 815-27.
19. Marguerie GA, Plow EF, Edgington TS. Human platelets possess an inducible and saturable receptor specific for fibrinogen. J Biol Chem 1979; 254: 5357-63.
20. Peerschke EI, Zucker MB, Grant RA, Egan JJ, Johnson MM. Correlation between fibrinogen binding to human platelets and platelet aggregability. Blood 1980; 55: 841-7.
21. Marguerie GA, Plow EF. Interaction of fibrinogen with the platelet receptor: Kinetics and effect of pH and temperature. Biochem 1981; 20: 1074-80.
22. Frojmovic MM, Mooney RM, Wong T. Dynamics of platelet glycoprotein IIb-IIIa receptor expression and fibrinogen binding. I. Quantal activation of platelet subpopulations varies with adenosine diphosphate concentration. Biophys J 1994; 67: 2060-8.
23. Bell DN, Spain S, Goldsmith HL. The ADP-induced aggregation of human platelets in flow through tubes: I. Measurement of the concentration and size of single platelets and aggregates. Biophys J 1989; 56: 817-28.
24. Bell DN, Spain S, Goldsmith HL. The ADP-induced aggregation of human platelets in flow through tubes: II. Effect of shear rate, donor sex, and ADP concentration. Biophys J 1989; 56: 829-43.
25. Rochon YP, Frojmovic MM. Dynamics of human neutrophil aggregation evaluated by flow cytometry. J Leukocyte Biol 1991; 50: 434-43.
26. Smoluchowski, M von. Versuch einer mathematischen Theorie der Koagulationskinetik kolloider Lösungen. Z Phys Chem 1917; 92: 129-68.
27. Wong T, Pedvis L, Frojmovic, MM. Platelet size affects both micro- and macro-aggregation: contribution of platelet number, volume fraction and cell surface. Thromb Haemost 1989; 62: 733-41.
28. George JN, Onofre AR. Human platelet surface binding of endogenous secreted factor-VIII-von-Willebrand-factor and platelet factor 4. Blood 1982; 59: 194-7.
29. Azzam K, Cissé-Thiam M, Drouet L. The antithrombotic effect of aurin tricarboxylic acid in the guinea pig is not soley due to the interaction with the von Willebrand factor-GPIb axis. Thromb Haemost 1996; 75: 203-10.
30. Strony J, Phillips M, Brands D, Moake J, Adelman B. Aurin tricarboxylic acid in a canine model of coronary artery thrombosis. Circulation 1990; 81: 1106-14.
31. Parker RI, Gralnick HR. Identification of platelet glycoprotein IIb/IIIa as the major binding site for released platelet-von Willebrand factor. Blood 1986; 68: 732-6.
32. Lawrence MB, Springer TA. Leukocytes roll on a selectin at physiological flow rates: Distinction from and prerequisite for adhesion through integrins. Cell 1991; 65: 859-73.
33. Savage B, Saldivar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. Cell 1996; 84: 289-97.
34. Wagner CT, Kroll MH, Chow J, Hellums JD, Schafer AI. Epinephrine and shear stress synergistically induce platelet aggregation via a mechanism that partially bypasses vWF-GP-Ib interactions. Biorheology 1996; 33 (3): 209-29.

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