

**Role of fibronectin in platelet adhesion
and aggregation: impact of biomechanics and $\beta 3$
integrin on fibrillogenesis**

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Abbreviations

ADP	Adenosine diphosphate
APS	Ammonium persulphate
BSA	Bovine serum albumin
CaCl ₂	Calcium chloride
Cyto D	Cytochalasin D
DOC	Deoxycholate
EDTA	Ethylenediaminetetraacetic acid
Fg	Fibrinogen
Fn	Fibronectin
FRET	Fluorescence resonance energy transfer
GdnHCl	Guanidine hydrochloride
H ₂ O	water
Jas	Jasplakinolide
KCl	Potassium chloride
kDa	kilo Dalton
KH ₂ PO ₄	Monopotassium phosphate
Lat A	Latrunculin A
MgCl ₂	Magnesium chloride
Na ₂ HPO ₄	Sodium phosphate dibasic
NaCl	Sodium chloride
NaN ₃	Sodium azide
PBS	Phosphate buffered saline
PHSRN	Proline-histidine-serine-arginine-asparagine sequence
PMA	Phorbol 12-myristate 13-acetate
PMMA	para-Methoxy-N-methylamphetamine
PMSF	Phenylmethanesulfonyl fluoride

PRP	Platelet-rich plasma
Reopro	Abciximab antibody
RGD	Arginine-glycine-aspartic acid sequence
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEMED	N, N, N', N'-tetramethylethylenediamine
UV	Ultraviolet
vWF	von Willebrand factor
%	Percentage
° C	degree Celsius
µg	microgram
µM	micromolar
g	gram (weight)
L	Liter
M	Molar (= mol/L)
mg	milligram
ml	milliliter
nm	nanometer
rpm	revolutions per minute
s ⁻¹	inverse seconds

1. Introduction

1.1 Fibronectin (Fn)

1.1.1 Structure of Fn

Fn is a dimeric glycoprotein of 230-270 kDa subunits that is present in the extracellular matrix and in blood plasma [4-5]. Fn is a modular protein that comprises three types of repeating units: twelve type I repeats (FnI), two type II repeats (FnII) and 15-17 type III repeats (FnIII) [4, 6] (Figure 1.1). The type I and type II repeats contain two intramolecular disulfide bonds to stabilize their folded structure while the type III repeat is a 7-stranded β -barrel structure lacking disulfide bonds [7-9]. Therefore, the type III repeats can undergo conformational changes [10]. Sets of modules are organized into functional domains including the N-terminal 70 kDa domain, the 120 kDa central binding domain and the heparin-binding domain [1, 11]. The diverse set of binding domains allows Fn to interact with multiple cellular integrin receptors, collagen, gelatin (but not *in vivo*), heparin and other extracellular molecules including Fn itself [3].

The primary gene transcript of Fn can generate multiple mRNA transcripts leading to distinct Fn isoforms by alternatively splicing [11]. There are about 20 monomeric isoforms in humans and about 12 isoforms in rodents and cows [12]. Alternatively splicing occurs at three sites among the type III repeats: extra type III domains EIIIA/EDA (between III11 and III12), EIIB/EDB (between III7 and III8) and the V region/IIICS (between III14 and III15) [3]. Each of these splicing regions may carry out some unique functions of Fn regarding cell adhesive activities or protein solubility and stability.

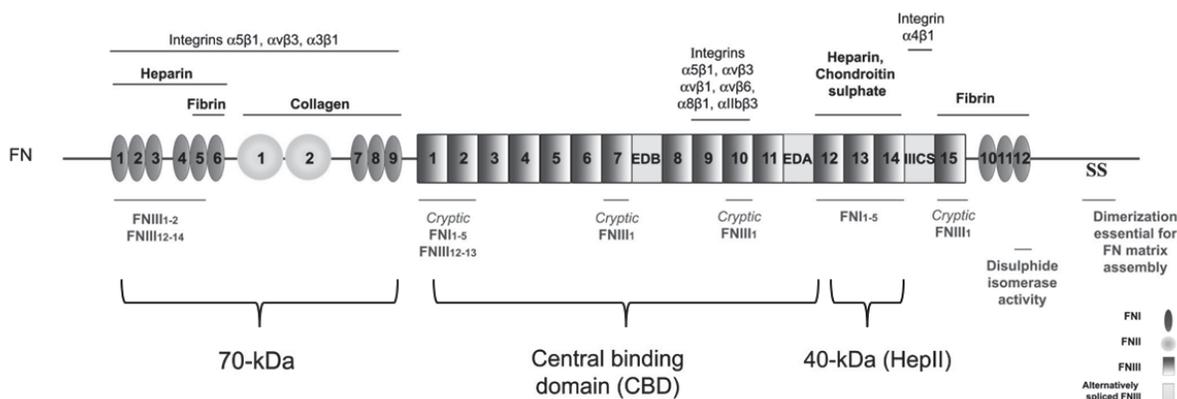


Figure 1. 1: The domain structure of Fn

Fn comprises three types of repeating units: twelve type I repeats (FnI), two type II repeats (FnII) and 15-17 type III repeats (FnIII). Set of modules are organized into functional domains including the N-terminal 70 kDa domain, the 120 kDa central binding domain and the heparin-binding domain. The three alternative spliced sites (EDA, EDB, and IIICS) are also shown. Figure modified from To et al [1].

1.1.2 Plasma Fn and cellular Fn

Fn exists in two major forms: plasma Fn and cellular Fn. Plasma Fn is produced by hepatocytes in the liver and is secreted into circulation at a concentration of 300-400 $\mu\text{g/ml}$ in a soluble, compact and non-fibrillar form [13]. Plasma Fn does not contain the extra domains EIIIA/EDA and EIIIB/EDB and has only one subunit that contains a V domain [14]. In contrast, cellular Fn is a mixture of Fn isoforms synthesized by many cell types including endothelial cells, chondrocytes, myocytes, synovial cells and fibroblasts [4]. The alternative spliced transcripts of Fn mRNA generate various isoforms of cellular Fn. They are expressed in a cell-specific and species-specific manner [15]. Therefore, this process has the capacity to produce a large number of Fn variants. These variants differ in solubility, ligand-binding capacity and cell-adhesive properties in order to provide a mechanism for cells to alter the composition of the extracellular matrix and create their specific micro-environment. Furthermore, the functions of these variants of Fn are to modulate cell adhesion, migration, growth and differentiation. Studies on the roles of plasma and cellular Fn during tissue injury and repair have indicated that these two forms of Fn possess distinct functions. Blood circulating plasma Fn has the tendency to function during early wound healing responses whereas cellular Fn is expressed and locally assembled during later wound-healing responses [1]. However, in some cases, plasma and cellular Fn could potentially perform the same function to compensate the loss of each. For instance, conditional plasma Fn knock-out mice using Cre-loxP system were shown to have normal skin-wound healing and hemostasis. This suggests that cellular Fn derived from platelets might be able to compensate for

the absence of plasma Fn [16]. In addition, plasma Fn was reported to diffuse into tissues and is incorporated into the fibrillar matrix [1]. In this study, I focus on plasma Fn because of its tendency to modulate early wound healing processes.

1.1.3 Major steps in Fn assembly

Intrinsic functions of Fn in the body are prevalent to the multimeric Fn fibrils that are components of the extracellular matrix. Plasma Fn will not form multimeric fibrils even at very high concentration (about 300 $\mu\text{g/ml}$ in human) to prevent the life-threatening effects [11, 17]. The process to incorporate soluble Fn into functional multimeric fibrils in the extracellular matrix is termed Fn fibrillogenesis or Fn assembly which is a stepwise, cell-mediated process [3] (Figure 1.2). Initiation of Fn matrix assembly depends on the binding of Fn dimers to cellular receptor integrins and subsequently conformational changes of the bound Fn. A dimeric Fn molecule binds to integrins, induces outside-in signaling [18] leads to integrin clustering which brings together bound-Fn dimers to promote Fn-Fn interactions. Therefore, the pair of cysteines at the C terminus that mediate the dimer structure of Fn is essential for the assembly process [19]. The binding of Fn to integrins induces formation of focal adhesion complexes where the cytoplasmic tails of integrins connect with the actin cytoskeleton [20]. The contractility of the cytoskeleton produced by actin-myosin filaments generates tension at contact sites between integrins and Fn [21-22]. Tethering of an Fn dimer on two integrins induces the cell contractility and applies forces to unfold Fn [23]. The conformational changes of Fn exposes the cryptic Fn-binding domains that are inaccessible in the compact form and allow them to contribute to Fn-Fn interactions [11]. These events lead to the formation of Fn fibrils by end-to-end association of Fn dimers [24]. Initial fibrils are first thin and DOC-soluble. The fibrils then grow in length and thickness and become an irreversible DOC-insoluble matrix [25].

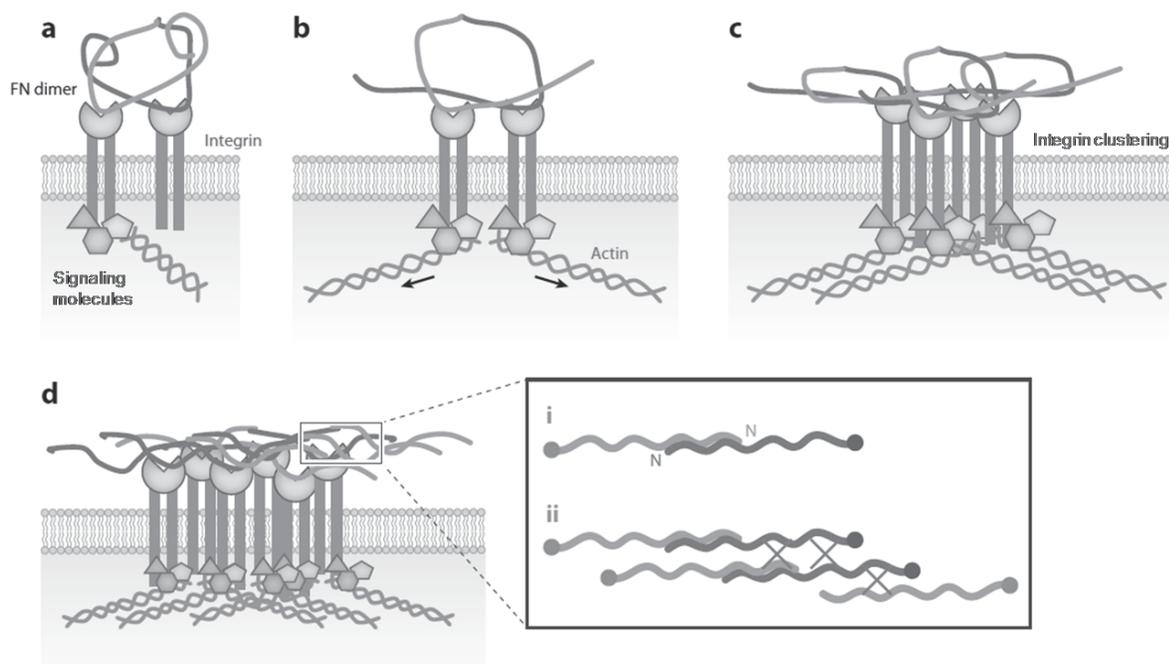


Figure 1. 2: Major steps in Fn assembly on platelet

Compact soluble Fn dimer binds to activated integrins on platelet (a). The interaction of integrin-fibronectin recruits signaling molecules that connect with actin cytoskeleton. Actin polymerization increases cell contractility that induces conformational changes in Fn to expose the cryptic Fn-binding domains (b). Integrin clustering brings unfolded bound-Fn dimers together to promote Fn-Fn interaction and further changes in Fn conformation (c). Finally, these events lead to the formation of Fn fibrils (d). Inset shows the Fn-Fn interaction during assembly. Fibrils form through end-to-end association of Fn dimers mediated by the N-terminal 70 kDa fragment (i). Lateral interactions between fibrils involve more other Fn binding sites (ii). Figure modified from Singh et al [3].

1.2 Integrins

Integrins are glycosylated, heterodimeric type I transmembrane receptors that are composed of non-covalently bound α - and β - subunits. Both subunits contain a large extracellular domain, a transmembrane domain and a short cytoplasmic tail [18]. The name integrin refers to the function of these molecules of linking the extracellular matrix with the intracellular cytoskeleton that is important in regulating biological processes such as cell proliferation, differentiation, adhesion, migration, etc. [26]. The combination of α - and β -subunit determines the ligand specificity, expression on the cell surface and intracellular signaling events of the integrins. In humans, 18 α - and 8 β -subunits had been described to form an integrin receptor family of 24 different heterodimeric members [18, 27]. Integrins are widely expressed on a variety of cells and most cells normally express several different integrins. Many integrins have the binding specificities for multiple ligands as well as a specific ligand can bind to more than one type of integrin. However, despite of the overlapping binding capacities, in most cases integrins can not

compensate for each. It is clear that intracellular signals generated by interaction with ligands are dependent on the type of integrin [28-29].

1.2.1 Fn receptors (integrins) on the platelet surface

Several members of the integrin receptor family can be the receptor for Fn ligands. They are integrins that contain the $\alpha 4$ -, $\alpha 5$ -, $\alpha 8$ -, αIIb -, αv - subunits. These integrin receptors support cell adhesion and migration on Fn substrates. But not all of them have the ability to assemble Fn into fibrils [1]. Among them, four integrins, $\alpha 5\beta 1$, $\alpha 4\beta 1$, $\alpha \text{IIb}\beta 3$, and $\alpha \text{v}\beta 3$ were reported to trigger Fn fibrillogenesis. Different experiments had suggested that in contrast to $\alpha 5\beta 1$, which is the primary receptor for Fn, the three latter integrins are not capable to assemble Fn into fibrils without additional agonist-mediated cell activation [10].

Platelets are anucleated, subcellular fragments derived from megakaryotes [30]. The fundamental physiological role of platelets is to ensure hemostasis to prevent blood loss upon vascular injury [31]. Platelets express five integrin α subunits and two β subunits on their surface to form the three $\beta 1$ integrins namely $\alpha 2\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$ and two members of the $\beta 3$ integrin family namely $\alpha \text{IIb}\beta 3$, and $\alpha \text{v}\beta 3$ [32] (Table 1.1). The integrin $\alpha 5\beta 1$, $\alpha \text{IIb}\beta 3$, and $\alpha \text{v}\beta 3$ are known to be able to assemble Fn fibrils and have been described to play a role in platelet function [33]. Integrin $\alpha \text{IIb}\beta 3$ in particular, is the major receptor on platelet surface with the expression of about 80.000 copies/platelet and plays a key role in platelet adhesion and aggregation. Its biological importance is reflected by the fact that its loss or dysfunction in individuals such as Glanzmann thrombosthenia patients causes defects in platelet aggregation and subsequent bleeding disorders [32]. Although the most important function of $\alpha \text{IIb}\beta 3$ is to bind fibrinogen during hemostasis and thrombosis, it is able to recognize Fn and other RGD-containing ligands which are probably physiologically relevant for hemostasis [34-35]. In contrast, $\alpha \text{v}\beta 3$ is of minor receptor expressed on platelets. The expression level of $\alpha \text{v}\beta 3$ had been reported to be only a few hundred copies on the platelet surface [32]. Despite of its low expression level, the 50% sequence homology in the α subunit suggests that $\alpha \text{v}\beta 3$ is structurally similar to $\alpha \text{IIb}\beta 3$ [36]. In fact, $\alpha \text{v}\beta 3$ can recognize almost the ligands for $\alpha \text{IIb}\beta 3$ and is reported to contribute to platelet adhesion. Nevertheless, there are notable differences between $\alpha \text{IIb}\beta 3$ and $\alpha \text{v}\beta 3$ both structurally and functionally. The differences in the α subunit as well as the glycosylation in β subunit between these two $\beta 3$ integrins may account for some of the differences in activation, cation sensitivity and preferred ligand binding activity [32].

Integrin	Number of copies	Ligands
The $\alpha 2\beta 1$	2000 - 4000	Collagen
The $\alpha 5\beta 1$	2000 - 3000	Fn
The $\alpha 6\beta 1$	2000 - 3000	Laminin
The $\alpha \text{IIb}\beta 3$	about 80000	Fn, Fg, vWF, vitronectin, thrombospondin
The $\alpha \nu\beta 3$	few hundred	Fn, vWF, vitronectin, thrombospondin

Table 1. 1: Platelet integrin receptors and their main adhesive ligands. (modified from Cho et al. [37])

1.2.2 Integrin activation

The ligand-binding pocket of integrins is formed by the globular head of both subunits. In the absence of a ligand or agonist, bonds between the rest of the extracellular domains and cytoplasmic tails hold the head in the “bent” conformation. This “bent” conformation is preferred to an inactive form that has a low affinity to ligands [38]. Several observations had indicated that $\alpha \text{IIb}\beta 3$ and $\alpha \nu\beta 3$ must undergo conformational changes in the extracellular domains to shift to a high affinity state in the active form [39]. Transitions between the two states are dynamically regulated by bi-directional (inside-out and outside-in) signals (Figure 1.3). Platelet activation by physiological agonists like ADP or thrombin induces inside-out signaling and the binding of cytosolic proteins to the cytoplasmic tail of $\beta 3$ integrins and in turn triggers conformational changes in the extracellular ligand-binding head. In outside-in signaling, extracellular matrix proteins bind to the head of $\beta 3$ integrins and triggers conformational changes that are transmitted to cytoplasmic tails to allow them to interact with intracellular proteins to regulate cell functions [18]. These two processes are often linked in a synergistically manner. Integrin activation by inside-out signaling increases ligand binding that causes outside-in signaling. Outside-in signaling generated by ligand binding in turn induces intracellular signals that lead to inside-out signaling [2].

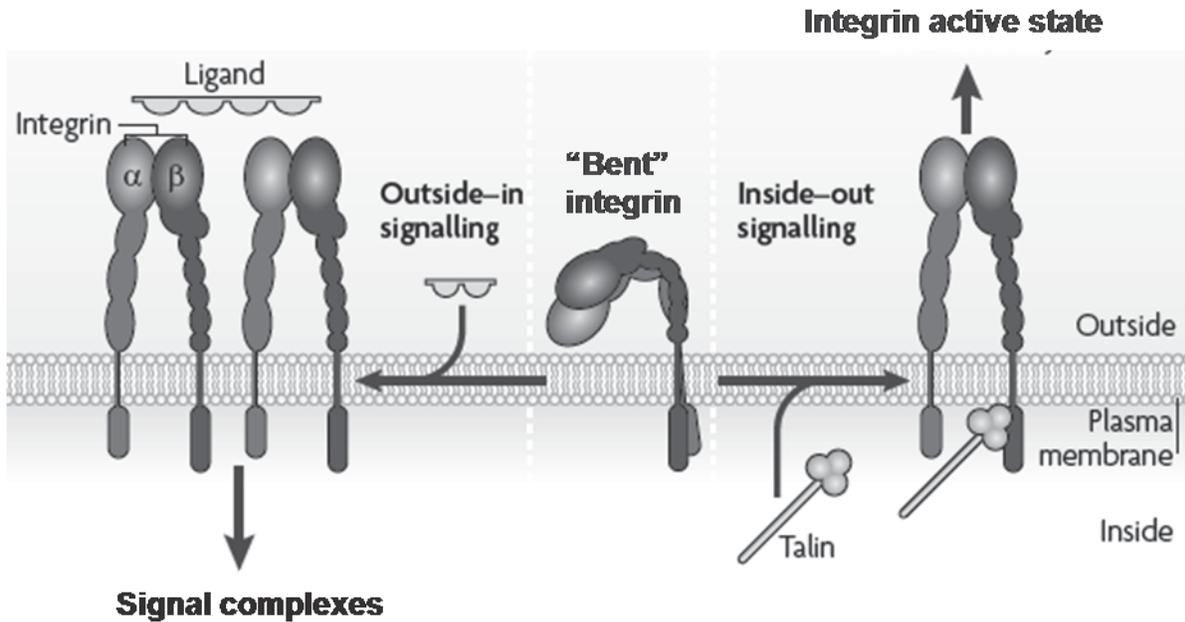


Figure 1. 3: Integrin activation through bi-directional signaling

In the absence of ligands or agonists, integrins are in their “bent” conformation representing an inactive state. Transition of integrins from inactive to active state is dynamically regulated by bi-directional signaling. In inside-out signaling, cytosolic proteins such as Talin or Kindlin bind and sequester the cytoplasmic tail of β_3 integrins and in turn trigger conformational changes in the extracellular ligand-binding head. In outside-in signaling ligands bind to the head of β_3 integrins trigger conformational changes that are transmitted to cytoplasmic tails to allow them to interact with intracellular proteins and regulate cell functions. Figure modified from Shattil et al [2].

1.2.3 Fn-integrin interaction during fibril assembly

The mechanism of how Fn becomes unfolded and assembled by interacting with cellular receptor integrins has not been well understood to date. It has been suggested that the process is dependent on interactions of more than one region within the Fn molecule to more than one type of integrin [3]. Fn is thought to bind initially to the yet unknown receptors on the cell surface via the 70 kDa N-terminal domain [40-44]. After this initiation, essential steps in the progression of Fn fibrils assembly involve the binding of the RGD motif within the domain FnIII10 and the neighboring PHSRN sequence in domain FnIII9 (see Figure 1.1) with integrins [1]. β_3 integrins ($\alpha v\beta_3$ and $\alpha IIb\beta_3$) which are expressed on platelets have been reported to interact with the RGD loop of Fn and to be involved in Fn fibril assembly [33].

1.3 Fn in platelet functions in hemostasis

The plasma Fn was first discovered as a contaminant of purified Fg. Subsequently, pFn was demonstrated to be incorporated in fibrin clots catalyzed by factor FXIIIa [45]. Such cross-linking alters property and the structure of the fibrin network [45-47]. This is the first evidence that plasma Fn has a potential hemostatic function.

1.3.1 Fn in platelet adhesion

Platelet adhesion at sites of exposed extracellular matrix following vascular injury is the initial and crucial step in hemostasis [48]. There are many factors that can affect platelet adhesion on extracellular matrix proteins. First, platelets do not adhere equally to all of extracellular matrix components. Second, since platelets have to perform their function in an environment that involves a constant fluid motion, shear stress generated by different flow conditions is one of the modulators. Finally, platelet adhesion is also dependent on the depth and extent of the injury [30].

Different reports have suggested a role of Fn in platelet adhesion. By performing flow chamber studies, the group of Jan J. Sixma in the mid-1980s had shown that Fn is important for platelet adhesion on non-fibrillar collagen type I and III, extracellular matrix of cultured endothelial cells, and the subendothelium of the vessel wall [49-50]. Moreover, a Fn surface supports platelet adhesion under static and flow conditions. Platelet adhesion to Fn is independent on shear rate but less efficient than other surfaces such as vWF and collagens [51]. The role of Fn in platelet adhesion under shear conditions was further confirmed by different experiments showing that antibodies against Fn decrease platelet adhesion to subendothelial matrix [52].

1.3.2 Fn in platelet aggregation

In the last years, controversial data have been published that support Fn as either an enhancer or inhibitor of platelet aggregation. Plasma Fn had been showed as a determinant for thrombus formation on collagen, fibrin, or fibrin cross-linked by Fn [50, 53]. Moreover, by using plasma Fn-coated beads, Matuskova et. al. had shown that plasma Fn is deposited on developing thrombi formed under high shear conditions *in vitro* [54]. Mice with a conditional depletion of plasma Fn exhibit a delayed thrombus growth and the inability to form stable thrombi at sites of injury [55]. However, addition of exogenous plasma Fn to platelets in suspension had showed to decrease platelet aggregation by thrombin, collagen, and ionophore A23187 [56-57]. Recently, a study on

mice with triple depletion of vWF/Fg/Fn showed that platelet aggregation and thrombus formation were enhanced in comparison with Fg/vWF double depleted mice [58].

1.3.3 Fn assembly in platelet adhesion and aggregation

A potential explanation has been suggested for the contradictory role of Fn in platelet aggregation and thrombus formation. The compact soluble plasma Fn may act like an inhibitor but after transitioning into unfolded insoluble fibrils following interaction with platelet receptor integrins might act like an enhancer for platelet aggregation. While experiments are needed to be done to prove this hypothesis, there are some apparently satisfactory observations: 1) Fn is known to assemble into fibrillar networks on adherent platelets [59]. 2) The ability of adherent platelets to assemble Fn appeared to be dependent on the adhesive substrates. Platelets assemble Fn when adherent to Fn, fibrin, laminin 111, and collagen type I but this was prevented when they adhere to Fg and vitronectin [33]. 3) Of note, whenever there is Fn assembly on adherent platelets there is an enhancement in thrombogenicity [53, 60]. 4) Fn assembly into fibrillar matrix supports better platelet adhesion compared to soluble Fn [61]. These observations may reflect the importance of Fn assembly for its function in platelet adhesion and aggregation. However, this hypothesis remains to be proved.

1.4 Description and importance of the present studie

Platelet adhesion and aggregation disorders are the leading cause of death in Western countries [62]. Therefore, understanding the molecular mechanisms of platelet-extracellular matrix protein interactions during hemostasis is of great importance to improve treatments for hemostatic diseases. Plasma Fn has been long suspected to play a role in hemostasis and thrombosis due to its high concentration in blood and its interaction with platelets [63]. However, its role in hemostasis and thrombosis is controversial and inconclusive. I hypothesize that there are differences in function between folded soluble Fn and unfolded insoluble Fn fibril leading to its dual role in platelet adhesion and aggregation. Consequentially, the goals of my studies can be divided into two main parts:

1. To examine the role of Fn in hemostasis and to establish the relationship between its molecular structure and its role
2. To investigate factors that can affect the conformational change of Fn leading to its role in hemostasis

I found that plasma Fn can play a dual effect in platelet adhesion and aggregation by inhibiting platelet aggregation but promoting platelet adhesion. To find out an explanation for this finding, my further studies focused on the interaction of Fn with suspended and adherent platelets. My data revealed that adherent but not suspended platelets induce conformational changes of Fn which are necessary for its assembly into fibrils. Hence, these observations can be used to explain for the dual role of Fn in hemostasis. Beyond that, it is necessary to elucidate the functions of Fn assembly during platelet adhesion. Therefore, I characterized the influences of actin polymerization, β_3 integrins, and shear stress conditions on Fn conformational changes during its interaction with adherent platelet. Fn unfolding and assembly is strongly influenced by actin polymerization as well as shear stress. $\alpha v\beta_3$ and $\alpha IIb\beta_3$ appeared to contribute equally to induce conformational changes in plasma Fn on adherent platelets under static condition despite the fact that $\alpha v\beta_3$ is of minor expression on platelet surface. Under high shear stress conditions, $\alpha v\beta_3$ is more important for the initiation of the Fn assembly process whereas $\alpha IIb\beta_3$ acts more intensively in the later phase of process progression.

2. Materials and Methods

2.1 Materials

All chemicals otherwise not mentioned here were from major suppliers.

2.1.1 General equipment and kits

Autoclave (3870 ELV, Tuttnauer), Centrifuge (5415R, Eppendorf), Centrifuge (Hettich universal 30RF), DiaMed Impact R viscometer (DiaMed), Diamed Impact R kit (Diamed), Dry Block Heater (Model 5436, Eppendorf), Electrophoresis apparatus (Bio-rad), Fluoroskan Ascent Microplate Fluorometer (Thermo Scientific), Genesys 10S UV/VIS Spectrophotometer (Thermo Scientific), Haematology analyzer (KX-21N, Sysmex), Incubator (Heraeus), Light transmission aggregometry (Dyasis Greiner), LS55 fluorescence spectrometer (Perkin-Elmer), Magnetic stirrer (MR3001K, Heidolph), Milligram balance (LA1200S, Sartorius), Molecular imager Chemidoc XRS (Bio-rad), pH meter (pH540GLP, Multical), Power pac universal (Bio-rad), Rotator (RM Multi-1, Star Lab), Water bath (model SW-20C, Julabo).

2.1.2 General chemicals and materials

30 % Acrylamide/ 0.8 % Bisacrylamide (National Diagnostics), ADP (Sigma), APS (Sigma), Apyrase (Sigma), BSA (Sigma), CaCl₂ (Merck), Coomassive Brilliant Blue R-250 staining solution (Bio-rad), Cyto D (Sigma), Dextrose (Sigma), EDTA (Merck), Fatty acid-free albumin (Sigma), Fresh frozen plasma (Blood center, University of Duesseldorf), GdnHCl (Sigma), Gelatin sepharose (Sigma), Glacial acetic acid (Merck), Glycerol (Roth), Glycine (Roth), HEPES (Sigma), Jas (Sigma), KCl (Merck), Lat A (Sigma), Methanol (Sigma), MgCl₂.6H₂O (Merck), NaCl (Merck), NaH₂PO₄ (Merck), NaHCO₃ (Merck), NaN₃ (Merck), PMA (Sigma), PMSF (Sigma), Protein inhibitor cocktail (Roche), Protein ladder (Bio-rad), SDS (Bio-rad), Sodium deoxycholate (Sigma), TEMED (Sigma), Tris base (Sigma), Urea (Sigma)

2.1.3 Antibodies, ligands and fluorescence dyes

Abciximab (Reopro, Lilly), Alexa Fluor 488 succinimidyl ester (Molecular Probes), Alexa Fluor 546 maleimide (Molecular Probes), Collagen (Sigma), Human fibrinogen (Sigma), Human Fn (Calbiochem), LM609 (Millipore)

2.1.4 Other materials

96-well plate (Costar, Corning Incorporated), blood collection set (BD vacutainer), blood collection tube (BD vacutainer), PD-10 desalting column (GE healthcare), Rotilabo® PMMA disposable cuvettes (Carl Roth GmbH), Sephadex G-25 gel filtration columns (GE healthcare), Slide A Lyzer dialysis device (Thermo Scientific)

2.1.5 Buffer and SDS-PAGE gel compositions

- PBS buffer: 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.3
- HEPES Tyrode's buffer: 136.5 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂·6H₂O, 3.3 mM NaH₂PO₄·H₂O, 10 mM HEPES, 5.5 mM dextrose and 1 g/l fatty acid-free albumin, pH 7.4
- 2% DOC lysis buffer: 2 % sodium deoxycholate, 20 mM Tris-Cl, pH 8.8, 2 mM PMSF, 2 mM EDTA and 1 tablet of protein inhibitor cocktail
- 1% SDS solubilization buffer: 1 % SDS, 20 mM Tris-Cl pH 8.8, 2 mM PMSF, 2 mM EDTA and 1 tablet of protein inhibitor cocktail
- SDS-PAGE running buffer (10X): 30 g Tris-base, 142 g glycine and 10 g SDS dissolved in 1 L of double distilled water.
- Destaining buffer: 100 ml Methanol, 100 ml glacial acetic acid and 800 ml H₂O distilled water
- Separating gel (6 % acrylamide): 3 ml Acrylamide/Bisacrylamide, 3.75 ml 4X Tris HCl/SDS pH 8.8, 0.1 ml APS 10%, 0.01 TEMED and 8.25 ml distilled H₂O.
- Stacking gel (3.9 % acrylamide): 0.65 ml Acrylamide/Bisacrylamide, 1.25 ml 4X Tris HCl/SDS pH 6.8, 0.05 ml APS 10%, 0.005 TEMED and 3.05 ml distilled H₂O.

2.2 Methods

2.2.1 Isolation of plasma Fn

Human plasma Fn was isolated by a modified procedure from previously described gelatin-sepharose chromatography [64]. Briefly, frozen human plasma obtained from the HHU Blood Center of Dusseldorf was thawed at 37 °C, and supplement with 10 mM EDTA and 0.02 % NaN₃. Plasma was then applied to a gelatin-sepharose packed column. The column was washed with 50 mM Tris pH 7.5 buffer until there was no detectable protein in the eluant (absorbance at 280 nm). Washing was continued with 1 M NaCl followed by 1 M urea. Finally, Fn was eluted by 3 M urea and immediately subjected to dialysis overnight in PBS pH 7.3 with 10 % glycerol to remove Urea. Its purity and concentration was examined by SDS-PAGE and UV measurement at 280 nm, respectively.

2.2.2 Platelet preparation

Human blood from healthy adult volunteers was collected into acid-citrate-dextrose containers. Whole blood was subjected to centrifugation at 1500 rpm for 10 min to obtain platelet-rich-plasma (PRP). 2 ml PRP were then transferred to a plastic tube containing 2 ml PBS pH 6.5 and 2.5 U/ml apyrase followed by centrifugation at 2500 rpm for 6 min. Platelet pellet was obtained and rested in 500 µl of HEPES Tyrode's buffer for 10 min at room temperature. Washed platelets were resuspended and platelet number was counted by using Haematology analyzer. Finally, platelet suspension was adjusted to 2.5×10^8 /ml with HEPES Tyrode's buffer. Addition of CaCl₂ 2 mM was done prior to the experiments.

2.2.3 Platelet aggregation assay

Washed platelets (2.5×10^8 /ml) were mixed with 300 µg/ml plasma Fn. Aggregation was induced by 40 nM PMA or 10 µg/ml collagen. In parallel experiments, Fg 3 mg/ml was added to platelet suspensions prior to the aggregation assays. For control experiments, washed platelets alone were tested for aggregation in the absence or presence of activated agonists (40 nM PMA or 10 µg/ml collagen). Platelet aggregation was monitored by recording changes in light transmission with the use of an aggregometer.

2.2.4 Platelet adhesion assay

Collagen type I, fibrinogen, and Fn (10 $\mu\text{g/ml}$) were coated onto wells of 96-well plates. Protein coated wells were subsequently blocked with 1 % BSA. Washed platelets ($10^7/\text{ml}$) were labeled with mepacrine (10 μM) for 1 h at room temperature. HEPES-Tyrode's buffer (200 μl) containing 2×10^6 mepacrine-labeled platelets and 2 mM CaCl_2 was added and incubated for 30 min at 37°C in the absence or presence of 100 $\mu\text{g/ml}$ plasma Fn. Unbound platelets were washed out three times with PBS buffer. Adherent platelets were quantified based on the fluorescence signal of mepacrine recorded by a microplate fluorometer (Fluoroskan Acent, Thermo scientific).

2.2.5 Fn labeling for FRET (Fluorescence resonance energy transfer)

Isolated plasma Fn was doubly labeled with alexa fluor 488 succinimidyl ester (donor) and alexa fluor 546 maleimide (acceptor) (Molecular probe, Invitrogen) for FRET experiments, as previously described [24]. Briefly, isolated Fn was denatured in 4 M GdnHCl to expose the two cryptic free cysteine residues. Alexa fluor 546 was then added to the protein solution at a molar ratio of 30:1 (dye/Fn molecule) to specifically label four free cysteine residues on Fn dimer molecule. The reaction was incubated in dark for 1 h at room temperature with a gentle rotation. After that, unbound dyes were removed by dialysis the sample with PBS pH 7.3 overnight. Alexa fluor 546 conjugated Fn (Fn546) protein was collected and concentration was measured by reading the absorption at 280 nm. Next, sodium bicarbonate at final concentration of 0.1 M pH 8.7 was added to the Fn546 solution for amine labeling according to the user manual. Labeling was done by adding 80-fold molar excess of alexa fluor 488 to the Fn546 solution and incubated for 1 h in dark at room temperature with gentle rotation. Free dyes were again removed by dialysis with PBS pH 7.3 overnight. Concentration and ratios of dye/Fn molecule conjugation were determined by reading the absorption at 280 nm, 496 nm and 556 nm and calculating according to the user manual. Batches of doubly labeled Fn which were conjugated with 3-4 alexa fluor 546 and 6-9 alexa fluor 488 were chosen for further experiments.

2.2.6 Sensitivity of FRET to changes in Fn conformation

To confirm the sensitivity of FRET to indicate changes of Fn conformation, labeled Fn was exposed to solutions of increasing concentration of GdnHCl 0-4 M and fluorescence signals were measured at 517 nm (donor emission wavelength) and 570 nm (acceptor emission wavelength)

with excitation wavelength at 488 nm by a LS55 fluorescence spectrometer (Perkin Elmer). FRET signals were determined as the ratio of acceptor fluorescence intensity divided by donor fluorescence intensity.

2.2.7 Fn unfolding by platelets monitored by FRET

Labeled Fn was mixed with at least 10-fold excess of unlabeled Fn to prevent energy transfer between adjacent protein molecules. For suspended platelets, a 100 $\mu\text{g/ml}$ of Fn mixture and 40 nM PMA were added to a solution of washed platelets ($10^8/\text{ml}$) in HEPES Tyrode's buffer supplemented with 2 mM CaCl_2 . Gentle stirring was applied to ensure that platelets were kept suspended. For adherent platelets, washed platelets ($10^8/\text{ml}$) in 2 ml of HEPES Tyrode's buffer supplemented with 2 mM CaCl_2 were allowed to adhere onto surfaces of 50 $\mu\text{g/ml}$ Fn-precoated PMMA cuvettes for 1 h at 37°C. Unbound platelets were removed by three times washing with PBS buffer and 10 $\mu\text{g/ml}$ of Fn mixture in HEPES Tyrode's buffer supplemented with 2 mM CaCl_2 and 40 nM PMA was added to the cuvettes. In both settings, FRET signals were recorded after 0 h, 1 h, 2 h and 3 h of incubation. For control experiments, FRET signals of a Fn mixture without platelets were also recorded. For experiments studying the contribution of β_3 integrins in Fn unfolding, antibody LM609 (blocking $\alpha_v\beta_3$, 5 $\mu\text{g/ml}$) or 10E5 (blocking $\alpha_{IIb}\beta_3$, 10 $\mu\text{g/ml}$) or ReoPro (blocking β_3 integrins, 10 $\mu\text{g/ml}$) was incubated with adherent platelets for 15 min at room temperature prior to the addition of the Fn mixture. For experiments studying the effect of cytoskeleton drugs on Fn unfolding, platelets adherent onto Fn surfaces were pre-incubated with Cyto D (1 μM and 10 μM) or Lat A (40 nM and 400 nM), or Jas 1 μM for 15 min at room temperature before adding Fn mixture for FRET analyses.

2.2.8 DOC-solubility assay to study Fn assembly by adherent platelets under flow conditions

Polystyrene plates were coated with 50 $\mu\text{g/ml}$ Fn at 37 °C for 1 h. Alexa fluor 488 conjugated Fn (Fn488) was added to suspensions of washed platelets ($10^8/\text{ml}$) in HEPES Tyrode's buffer containing 2 mM CaCl_2 and 10 μM ADP. The mixtures (150 μl) were then applied onto Fn pre-coated polystyrene plates and immediately cover with the cones. DiaMed Impact-R device was used to generate flow conditions at shear rates of 500 s^{-1} and 5000 s^{-1} for 2 min and 10 min. After that, unbound platelets and molecules were removed carefully with pipette. Plates were washed

gently three times with PBS buffer followed by the addition of 150 μ l of 2 % DOC lysis buffer. Lysates were collected and total protein concentrations were determined by Bradford assay. The DOC-insoluble pellets containing Fn fibrils were isolated by centrifugation at 13500 rpm for 20 min. Pellets were then solubilized by using 100 μ l of 1 % SDS solubilization buffer. Equal amounts of insoluble samples based on total protein concentrations were loaded onto wells of 96-well microplates. Fluorescence signals from Fn488 of samples were recorded by using Fluoroskan microplate reader to compare the amount of Fn assembly. In some experiments, Reopro (10 μ g/ml) or LM609 (5 μ g/ml) was added to platelet mixtures before loading onto Fn precoated plates to study the contribution of β 3 integrins on the assembly of Fn under flow conditions.

2.2.9 Statistical analysis

All data were collected from at least three different experiments. Data were analyzed using GraphPad Quickcals (GraphPad software Inc., San Diego, CA, USA). To measure statistical differences, a t-test was used. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1 Purification of Fn from human plasma

To obtain a large and pure amount of Fn, outdated human plasma was collected from the blood center of HHU of Duesseldorf and the so-called gelatin affinity chromatography method was used. After loading the protein onto gelatin-sepharose column, non-specifically retained serum albumin and other proteins as well as Fg were removed by three washing steps using equilibration buffer (PBS pH 7.3), 1 M NaCl and 1 M Urea as shown in Figure 3.1A. Higher concentration and stronger denaturant gives larger protein yield, however, it can irreversibly damage the Fn structure. Therefore, Fn was eluted from the column by using 3 M Urea which is sufficient to break the Fn-gelatin interaction while preserving protein refolding properties. Isolated Fn in 3 M Urea buffer was immediately subjected to dialysis to remove Urea. SDS-PAGE by using 7.5 % polyacrylamide gel showed that the isolated Fn proteins exhibited a high purity since only a single band at 250 kDa that is typical for Fn under reducing condition was visible on the SDS-PAGE gel (Figure 3.1B). There were no bands of smaller fragments or bands of Fg to be detected by naked eyes as compared with commercial Fn and Fg (Figure 3.1C).

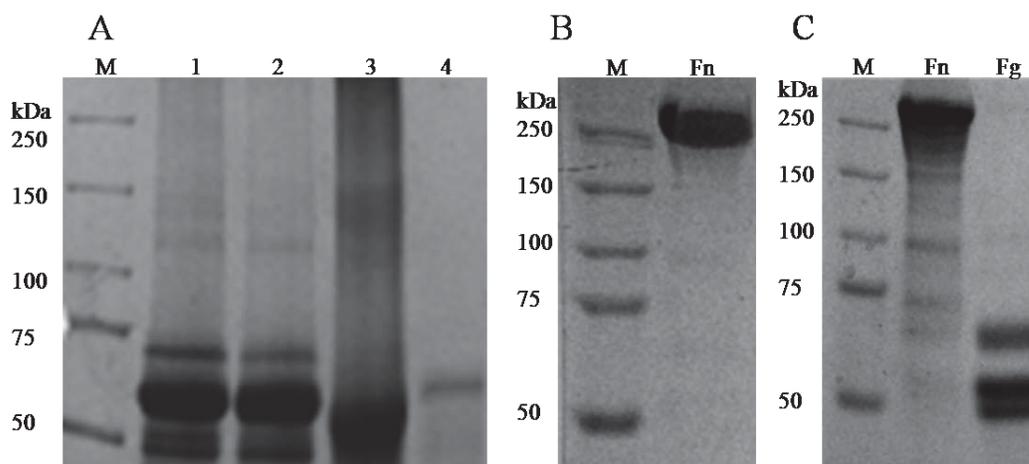


Figure 3.1: Purity of isolated Fn from human plasma assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

- (A) SDS-PAGE at different stages of the Fn isolation procedure (M) Protein marker, (1) Flowthrough, (2) Washed with Tris-Cl pH 7.5, (3) Washed with 1M NaCl, (4) Washed with 1 M Urea
- (B) and (C) SDS-PAGE of isolated Fn (B), commercial Fn and Fg (C) . 50 μ g of isolated Fn, commercial Fn and Fg were separated by 7.5 % SDS-PAGE under reducing conditions to confirm the purity. Gel was stained by Coomassie blue.

3.2 Fn enhances platelets adhesion but decreases platelet aggregation

Platelet adhesion and subsequently aggregation at sites of vascular injury are two crucial events to arrest bleeding. Therefore, to study the role of Fn in hemostasis, platelet adhesion and aggregation assays were performed in the presence or absence of exogenous plasma Fn.

3.2.1 Fn decreases platelet aggregation

The concentration of Fn in plasma is about 300-400 $\mu\text{g/ml}$ [65]. To test the effect of plasma Fn on platelet aggregation, isolated plasma Fn (300 $\mu\text{g/ml}$) was added to a solution of washed platelets ($2.5 \times 10^8/\text{ml}$) followed by the addition of 40 nM PMA or 10 $\mu\text{g/ml}$ collagen to induce platelet aggregation. ADP, another commonly used agonist for inducing platelet aggregation, was not chosen in our experiments because Fn does not bind to ADP-stimulated suspended platelets [66]. As shown in Figure 3.2A and 3.2B, addition of Fn to washed platelets resulted in a delay in the kinetic and a reduction of 25% or 50% in platelet aggregation induced by PMA or collagen, respectively. The suppressive effect of Fn on platelet aggregation was observed in a concentration dependent manner (supplemental data 1). In some experiments, the effect of Fn on platelet aggregation was examined in the presence of plasma Fg which is known to be a key molecule for this process. Adding a physiological concentration of 3 mg/ml Fg to platelet suspensions did not change the suppressive effect of Fn on platelet aggregation. Although the suppressive effects were less than experiments without the presence of Fg, a trend in delaying in the beginning and about 5-10 % reduction in the end of platelet aggregation process induced by PMA or collagen could be observed (Figure 3.2C and 3.2D). In control experiments where no agonists were added, platelets did not aggregate (Figure 3.2D).

3.2.2 Fn enhances platelet adhesion

To examine the effect of Fn on platelet adhesion, washed platelets were labeled with 10 μM mepacrine and subsequently allowed to adhere on 10 $\mu\text{g/ml}$ collagen, Fg or Fn in the presence of 100 $\mu\text{g/ml}$ plasma Fn and 10 μM ADP. The amounts of platelet adhesion were 0.32 ± 0.15 onto immobilized collagen, 0.26 ± 0.07 on to immobilized Fg, and 0.51 ± 0.05 onto immobilized Fn. Addition of soluble Fn increased the adhesion of platelets significantly up to 0.74 ± 0.13 ($p = 0.0112$, $n = 3$), 0.64 ± 0.13 ($p = 0.005$, $n=3$), or 1.02 ± 0.14 ($p = 0.0017$, $n = 3$) onto immobilized collagen, Fg, or Fn, respectively (Figure 3.3).

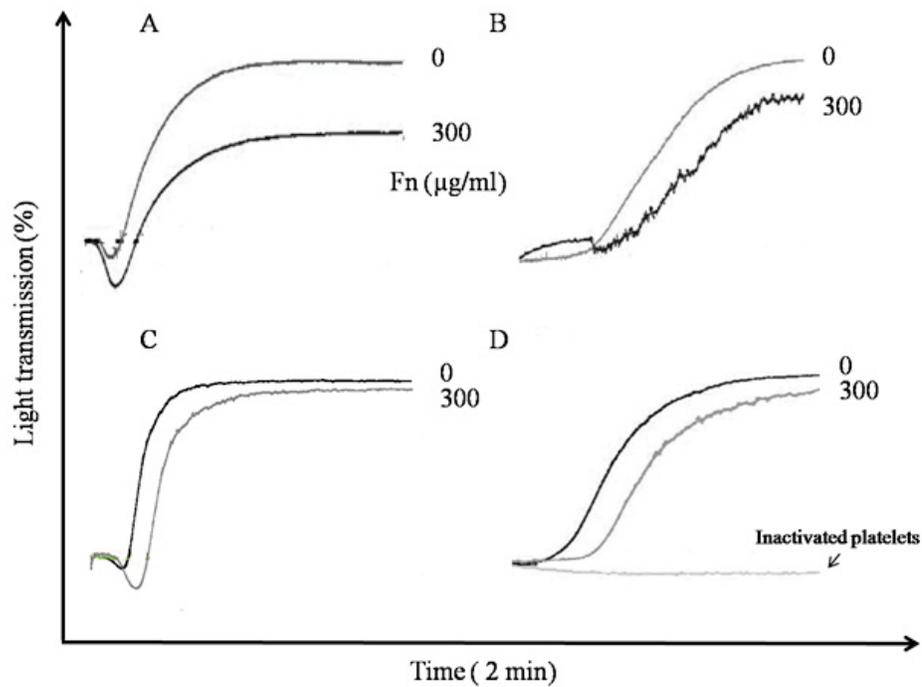


Figure 3.2: Fn decreased platelet aggregation measured by an aggregometer.

Washed platelets were stimulated with 10 µg/ml collagen (A) or 40 nM PMA (B) in the absence or presence of 300 µg/ml plasma Fn. In parallel experiments, Fg (3 mg/ml) was added to platelet suspensions prior to the aggregation activation by collagen (C) and PMA (D). Washed platelets did not aggregate without agonist (D).

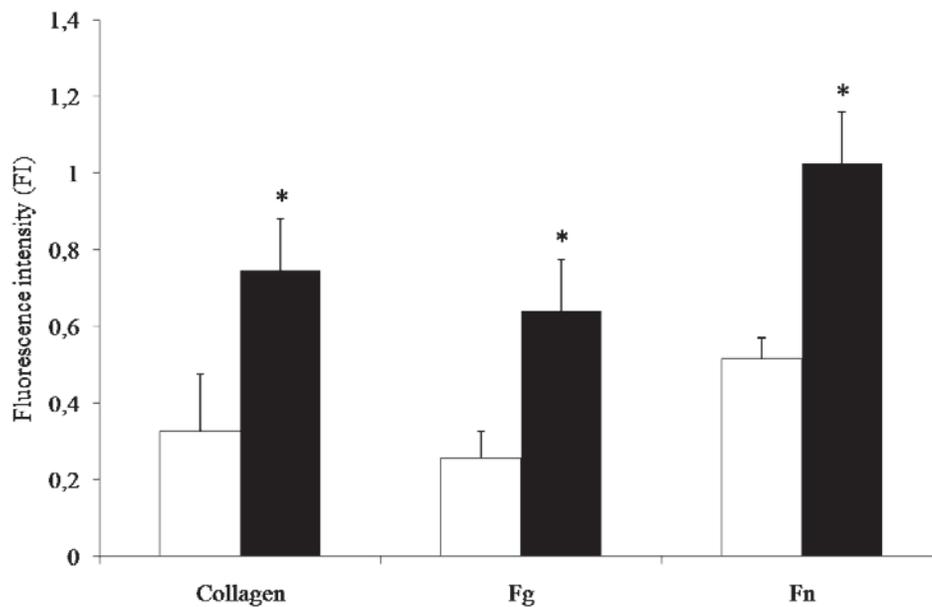


Figure 3.3: Fn enhanced platelet adhesion onto immobilized ligands

200 µl of mepacrine-labeled platelets (5×10^7 /ml) with (■) or without (□) 100 µg/ml plasma Fn in Tyrode's buffer containing 2 mM CaCl_2 were placed on protein-coated wells of 96-well plate and incubated for 30 min at 37°C. Adhesion was quantified by fluorescence intensity of mepacrine as described in "Materials and methods". Values represent the mean \pm SD of three individual experiments. (*) $p < 0.01$

3.3 Sensitivity of FRET to conformational changes of Fn in denaturing conditions

In vitro experiments showed that Fn can play a dual effect in hemostasis by decreasing platelet aggregation (Figure 3.2) and enhancing platelet adhesion (Figure 3.3). Because unfolding is a crucial process to turn Fn from its inactive to the active fibril form, it is assumed that the dual effect of Fn is due to the difference in Fn unfolding upon interacting with suspended platelets or adherent platelets. Therefore, FRET technology was applied as a tool to observe Fn unfolding *in vitro*. Isolated Fn was doubly labeled with alexa fluor 488 (donor) and alexa fluor 546 (acceptor) for FRET analyses by using two reaction chemistries: maleimide for coupling with cysteine residues and N-hydroxysuccinimidyl (NHS) ester for amine residues (Figure 3.4A). FRET signals are calculated as ratio of acceptor/donor fluorescence intensity. In principal, when Fn is in the soluble form with globular compact structure, the distances between donors and acceptors along the dimer molecule are shortest generating highest FRET signals. As Fn domains are extended when the protein is unfolded, the overall length of the dimer molecule is increased leading to the increase in average distance between donors and acceptors. The latter causes decreasing FRET signals. The more extended the molecules are, the lower FRET signals can be observed (Figure 3.4B).

To evaluate the sensitivity of FRET indicating the unfolding of Fn, labeled Fn was exposed to increasing concentrations of GdnHCl (1-4 M) and FRET signals were measured (scheme in Figure 3.4B). FRET signals decreased over the range of GdnHCl concentrations as shown in Figure 3.4C-3.4D indicating for the conformational changes of Fn from its compact to the unfolded state. FRET signals of Fn in its compact conformation (0 M GdnHCl) were shown as 100% and decreased to 64 % as it extended in 1 M GdnHCl solution. Further unfolding of Fn at 2 M, 3 M and 4 M concentrations of GdnHCl reduced the FRET signals to 50 %, 44 % and 40 %, respectively.

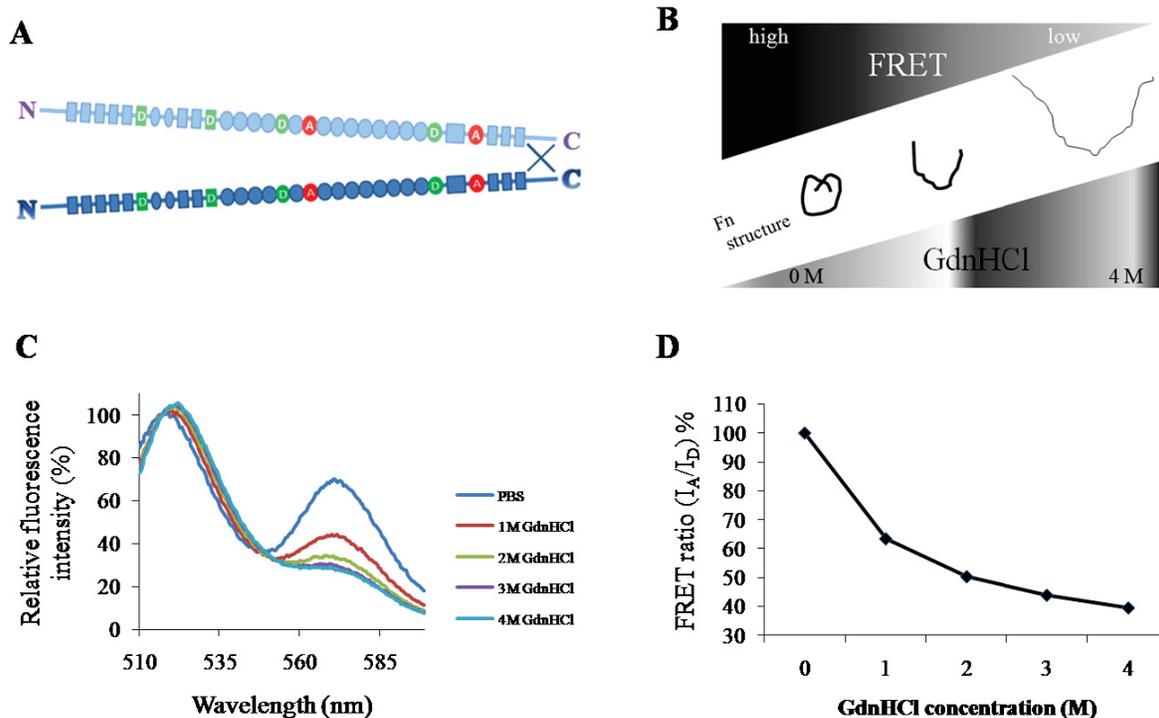


Figure 3. 4: Fluorescence conjugation and characterization of labeled Fn

- (A) Schematic sketch of the putative positions of fluorophores conjugated to Fn molecule for FRET. Fn was doubly labeled with alexa fluor 488 (donor) and alexa fluor 546 (acceptor) as described in “Materials and methods”.
- (B) Schematic sketch of Fn conformations in GdnHCl solutions correlated with FRET. Fn in solution of 0 M GdnHCl is in a compact structure so that it exhibits highest FRET signal. As GdnHCl concentration in solution increases to > 1 M or > 4M causing Fn to partially unfolded or unfolded, respectively, FRET signal decreases.
- (C) Fluorescence emission spectra of doubly labeled Fn exposed to solutions with increasing concentrations of GdnHCl. Spectra have been normalized to the donor peak so that changes in FRET are indicated solely by changes in the acceptor peak.
- (D) Reference curve probing Fn unfolding in GdnHCl solutions. Labeled Fn was exposed to 0 – 4 M GdnHCl and fluorescence intensities of donors and acceptors. FRET was calculated as the ratio of acceptor intensity divided by donor intensity (I_A/I_D). FRET of Fn in 0 M GdnHCl solution was shown as 100 %.

3.4 FRET analyses of Fn unfolding by platelets under static conditions

After confirming the sensitivity of FRET technology to detect Fn unfolding, labeled Fn was applied in further experiments to confirm the hypothesis that differences in Fn unfolding leads to its dual role in hemostasis. In addition, platelet factors that contribute to the process of unfolding of Fn molecules under static conditions were also studied.

3.4.1 Adherent but not suspended platelets progressively unfold Fn during interaction

To confirm the hypothesis, FRET-labeled Fn was incubated with PMA-stimulated platelets in suspension or platelets adherent onto immobilized Fn and changes in FRET signals were recorded within 3 h. Labeled Fn was mixed with at least 10 fold excess of unlabeled Fn to prevent the potential energy transfer between adjacent fluorescently labeled Fn molecules. Fn mixtures at final concentrations of 10 $\mu\text{g/ml}$ or 100 $\mu\text{g/ml}$ were incubated with adherent platelets or suspended platelets, respectively. For suspended platelets, a higher concentration of Fn was used because of the higher number of platelets and the larger interaction surface in comparison with adherent platelets. Long incubation times were chosen because of the presumably slow decrease of FRET signals due to excessive addition of unlabeled Fn and the secreted Fn from platelet granules. In addition, the small changes in FRET signal can be expected since the fluorescence signals are only partially from the cuvette walls. When labeled Fn was incubated with adherent platelets, FRET decreased in a time-dependent manner by $4 \pm 1.27\%$ at 1 h, $5 \pm 2.54\%$ at 2 h and $6 \pm 1.74\%$ at 3 h (Figure 3.5). To confirm that changes in FRET signals are caused by platelets on the inner wall of cuvettes after 3 h of incubation, samples were transferred to new cuvettes and FRET signals were measured. FRET signals of those samples showed only 1-2 % differences compared to FRET signals at the starting point. The slight increase of FRET signals in experiments over incubation time with labeled Fn alone might be due to the lower fluorescence stability of the donors compared to the acceptors. Moreover, a dynamic unfolding and refolding process might explain the observed oscillation of FRET signals in experiments where labeled Fn was incubated with suspended platelets. In general, there was no decrease in FRET signals of labeled Fn alone or incubated with suspended platelets over 3 h suggesting that Fn molecules did not change its overall conformation. Taken together, our data shows that activated adherent platelets but not suspended platelets progressively unfold soluble Fn during interaction.

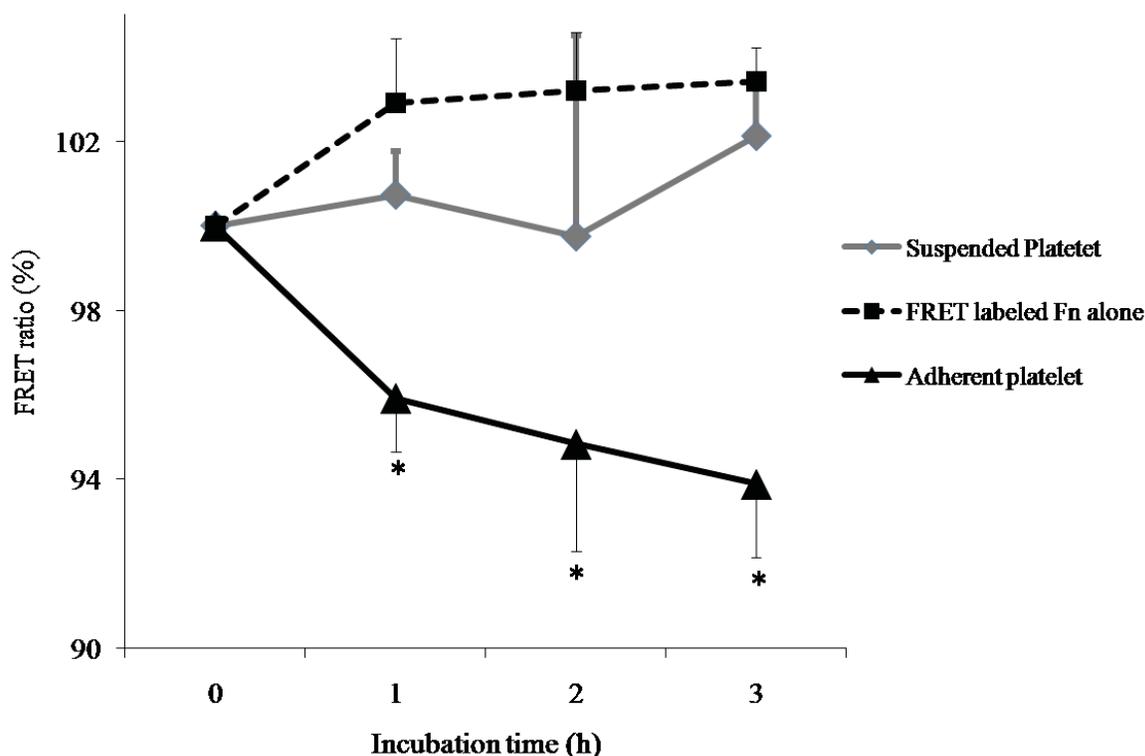


Figure 3.5: FRET measurement of Fn unfolded by platelets

Labeled Fn was mixed with at least 10 fold excess of unlabeled Fn to prevent energy transfer between adjacent protein molecules. Fn mixtures of 100 $\mu\text{g/ml}$ or 10 $\mu\text{g/ml}$ were incubated for 3 h at room temperature with washed platelets in suspension ($10^8/\text{ml}$) or with platelets adherent onto immobilized Fn (50 $\mu\text{g/ml}$), respectively. In both settings, platelets were stimulated with 40 nM PMA. As a control, FRET signals of Fn mixtures without platelets were also recorded by fluorescence spectrometer. FRET signals of all samples were shown as 100 % at the starting point (0 h of incubation). Values represent the mean \pm SD of three experiments. (*) $p < 0.05$

3.4.2 $\beta 3$ integrin-dependent unfolding of Fn during platelet adhesion under static conditions

Binding of $\beta 3$ integrins to the RGD motif and PHSRN sequence of Fn has been suggested to be crucial in progressing Fn unfolding and assembly [1]. To investigate the contribution of $\beta 3$ integrins in unfolding of Fn during platelet adhesion, adherent platelets on Fn were pre-incubated with 10 $\mu\text{g/ml}$ ReoPro (anti- $\beta 3$ antibody), or 5 $\mu\text{g/ml}$ LM609 (anti- $\alpha\beta 3$ antibody) prior to the addition of labeled Fn. Approximately 2×10^6 platelets adhered onto immobilized Fn of the inner wall of the cuvettes and did not detach when incubated with $\beta 3$ integrin antibodies (supplemental data 2). The decrease in FRET signals of labeled Fn was partially limited to 2.2 ± 0.23 , 2.8 ± 2.3 , 2.2 ± 0.08 % after 1, 2, 3 h of incubation, respectively, when platelet $\alpha\beta 3$ was blocked by

LM609. There was no decrease in FRET signals when adherent platelets were blocked with Reopro (Figure 6). These data indicate that Fn unfolding by adherent platelets onto immobilized Fn is dependent on $\beta 3$ integrins because Reopro can block both $\alpha \text{IIb}\beta 3$ and $\alpha \text{v}\beta 3$. Despite of the low expression level of $\alpha \text{v}\beta 3$ on platelets, it seems to share the same if not more contribution to Fn unfolding in comparison with $\alpha \text{IIb}\beta 3$.

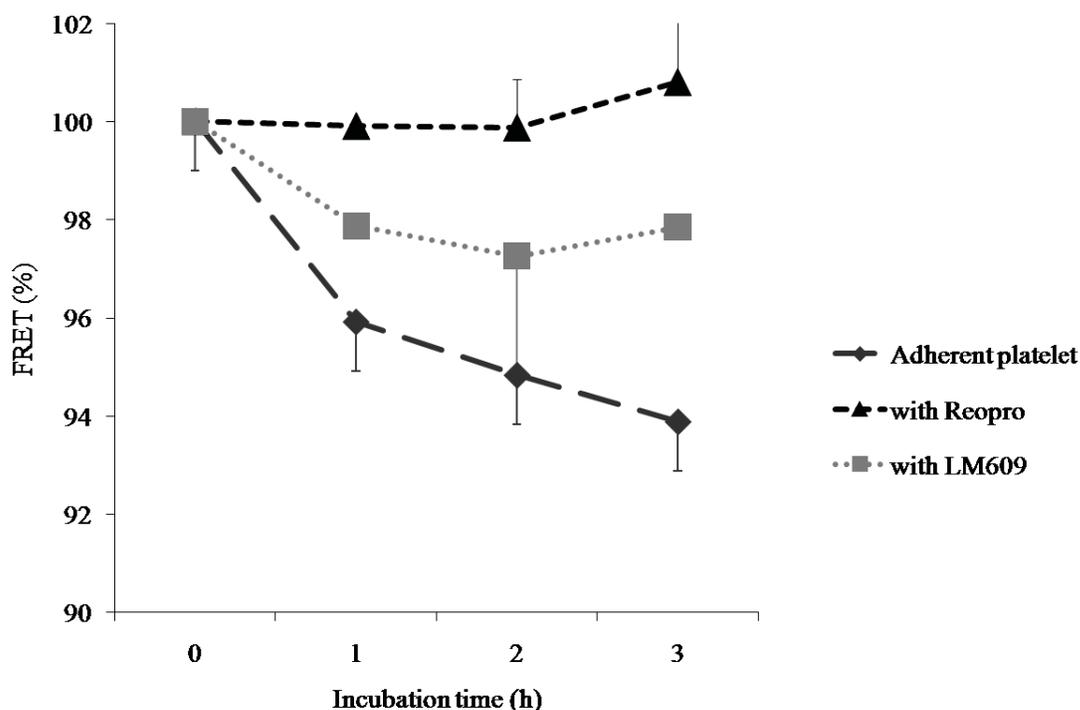


Figure 3. 6: Effects of $\beta 3$ blocking antibodies on Fn unfolding by adherent platelets

Platelets after adhesion on Fn-coated surface (50 $\mu\text{g/ml}$) were incubated with LM609 (blocking $\alpha \text{v}\beta 3$, 5 $\mu\text{g/ml}$) or Reopro (blocking $\beta 3$ integrin, 10 $\mu\text{g/ml}$) for 15 min at room temperature before adding 10 $\mu\text{g/ml}$ Fn mixture. Values represent the mean \pm SD of three experiments.

3.4.3 Effect of actin polymerization on Fn unfolding by adherent platelets under static conditions

Organization of the actin cytoskeleton is important to induce cell contraction that is transmitted through integrins to unfold Fn [20]. Therefore, Fn unfolding can be controlled by modulating the polymerization of actin filaments. To test this hypothesis, we used cytoskeleton drugs namely CytoD, LatA and Jas to modulate actin polymerization of platelets followed by the measurement of Fn unfolding. The two former drugs are used for inhibiting actin polymerization. CytoD is

known to bind to the end of actin filaments to prevent their elongation whereas LatA can bind to G-actin monomers and prevent them to cooperate into actin filaments. The latter drug, Jas, enhances actin polymerization by stimulating actin filament nucleation and stabilizing F-actin. Platelets after adhering onto immobilized Fn were treated with 1 μ M and 10 μ M CytoD or 40 and 400 nM LatA or 1 μ M Jas for 15 minutes before adding labeled Fn. After 1 h incubation of labeled Fn with untreated platelets and drug-treated platelets, FRET signals were measured. As shown in Figure 3.7, in the absence of cytoskeletal effectors, unfolding of Fn caused by adherent platelets on immobilized Fn led to a decrease of 4 ± 1.25 % in FRET signals. CytoD showed a concentration-dependent inhibition of Fn unfolding by platelets because at concentration of 1 and 10 μ M of CytoD, FRET decreases were limited to 3.96 ± 0.07 % or 0.7 ± 1.12 %, respectively. The same inhibitory effects were seen using LatA where FRET signal decrease was limited to 2.7 ± 1.12 or completely inhibited at concentrations of 40 nM and 400 nM, respectively. In contrast, treatment of adherent platelets with 1 μ M Jasplakinolide prior to the addition of labeled Fn caused decreases in FRET signals by 6.46 ± 0.5 % after 1 h of incubation showing that Fn unfolding was significantly enhanced under this condition. Taken together, the data showed that fibronectin unfolding is dependent on actin polymerization and therefore, maybe dependent on cellular forces.

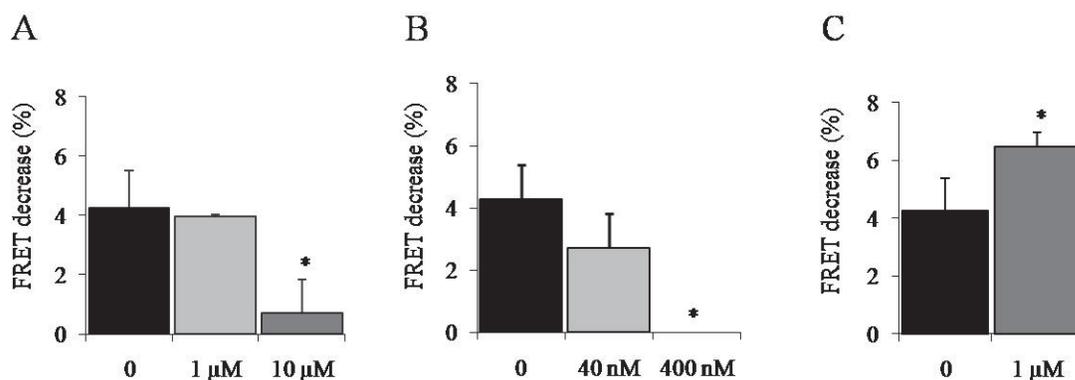


Figure 3.7: Effect of cytoskeleton effectors on Fn unfolding by adherent platelets after 1 h of incubation under static conditions

After adhesion on Fn-coated surface (50 μ g/ml), platelets were incubated with cyto D (1 or 10 μ M) (A) or Lat A (40 or 400 nM) (B) or Jas (1 μ M) (C) for 15 min at room temperature before adding 10 μ g/ml Fn mixture. Values represent the mean \pm SD of three individual experiments. (*) $p < 0.05$

3.5 Biomechanical stress modulates Fn unfolding by adherent platelets

Under *in vivo* conditions, platelets are constantly exposed to blood flow and their activity is affected by shear stress generated from blood flow [67]. There is evidence indicating that extracellular forces generated by shear stress is sensed by the integrins and transmitted to the actin cytoskeleton through mechanotransduction. Because FRET analyses showed that Fn unfolding is dependent on actin polymerization, I hypothesized that shear stress can modulate Fn unfolding by adherent platelets.

3.5.1 Fn assembly by adherent platelets under flow conditions

To examine the effect of shear stress on Fn assembly by adherent platelets, suspensions of platelets ($10^8/\text{ml}$) in Tyrode's buffer containing 2 mM CaCl_2 were placed on polystyrene plates (Diamed Impact-R test kit) precoated with 50 $\mu\text{g}/\text{ml}$ unlabeled Fn in the presence of 60 $\mu\text{g}/\text{ml}$ alexa488 conjugated Fn and 10 μM adenosine diphosphate (ADP). Two different flow conditions 500 s^{-1} and 5000 s^{-1} were generated for 2 and 10 min by using the cone and plate technology (Diamed Impact-R, Diamed). After that, adherent platelets were collected and the amount of fibril assembly on their surfaces was examined. Fn assembly by adherent platelets is observed to be strongly dependent on shear rate but not shear time (Figure 3.8). When shear rates increased from 500 s^{-1} to 5000 s^{-1} , there were significant increases in the amount of insoluble Fn detected on platelets after both shear times of 2 and 10 min suggesting that platelets under high shear rates assemble much more Fn fibrils on their surfaces (Figure 3.8A). However, increasing shear time from 2 to 10 min did not significantly alter the assembly of Fn by adherent platelets (Figure 3.8B). There were no insoluble fibrils that could be detected from adherent platelets under static conditions for 2 and 10 minutes.

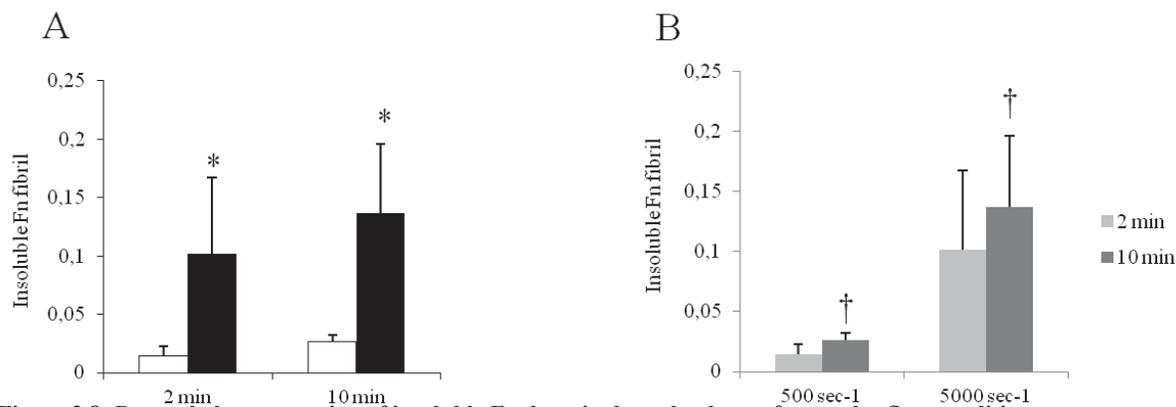


Figure 3.8: Deoxycholate extraction of insoluble Fn deposited on platelet surface under flow conditions.

Platelets were allowed to adhere in the presence of 60 $\mu\text{g/ml}$ Alexa488 conjugated Fn and 10 μM adenosine diphosphate (ADP) onto polystyrene plates coated with Fn under (A) different shear rates of 500 (\square) and 5000 s^{-1} (\blacksquare) or (B) different shear time for 2 (\blacksquare) and 10 min (\blacksquare). Adherent platelets were lysed with 2 % DOC lysis buffer. The DOC-insoluble fraction was separated by centrifugation at 13500 rpm for 20 minutes and solubilized by 1 % SDS. Equal volumes of DOC-insoluble fractions were analyzed for fluorescence signal. Values represent the mean \pm SD of three individual experiments. (†) $p =$ non significant, (*) $p < 0.05$

3.5.2 Effects of β_3 integrin antibodies on Fn unfolding by adherent platelets under flow conditions

β_3 integrins were observed to be important for adherent platelets to unfold Fn under static conditions. Therefore, it would be interesting to study their contribution to Fn assembly on platelets under flow. Thus, in parallel experiments, 10 $\mu\text{g/ml}$ ReoPro (anti- β_3 antibody), or 5 $\mu\text{g/ml}$ LM609 (anti- $\alpha_v\beta_3$ antibody) were added to suspensions of platelets in Tyrode's buffer containing 2 mM CaCl_2 before loading onto polystyrene plates (Diamed Impact-R test kit) precoated with 50 $\mu\text{g/ml}$ unlabeled Fn in the presence of 60 $\mu\text{g/ml}$ alexa488 conjugated Fn and 10 μM ADP. The same settings for generating flow conditions as described above were used. Under a shear rate of 500 s^{-1} , there were not significant insoluble Fn fibrils that could be detected from adherent platelets after 2 or 10 minutes (Figure 3.9A). After 2 min under higher shear rate of 5000 s^{-1} , platelets blocked with Reopro antibodies showed a significant decrease in the amounts of insoluble Fn fibril in comparison with control experiments where no antibody was used (Figure 3.9B). However, the same inhibitory effects could be seen for platelets treated with LM609 antibodies. In parallel experiments where 10 minutes of shear time were applied, both Reopro and LM609 antibodies showed an inhibitory effect on Fn fibrillogenesis with a stronger

inhibition when Reopro was used. Taken together, these data showed that $\alpha v\beta 3$ integrins even with the low expression on platelets but are more important than $\alpha IIb\beta 3$ in initiating Fn fibrillogenesis process under high shear rate conditions. The $\alpha IIb\beta 3$ integrins may contribute in the later steps in the progression of fibrils formation.

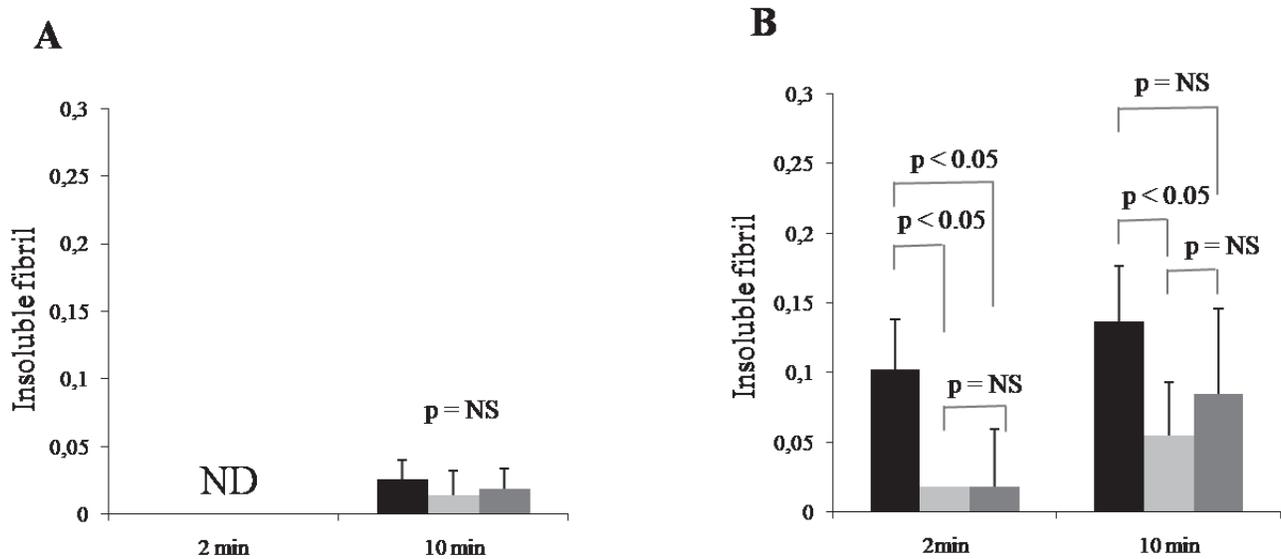


Figure 3. 9: Effect of $\beta 3$ blocking antibodies on Fn assembly by adherent platelets under flow conditions

LM609 (5 $\mu\text{g/ml}$) or Reopro (10 $\mu\text{g/ml}$) antibodies were added to platelets in suspension ($10^8/\text{ml}$) in Tyrode's buffer containing 2 mM CaCl_2 , 60 $\mu\text{g/ml}$ Alexa488 conjugated Fn and 10 μM ADP before loading onto polystyrene plates precoated with 50 $\mu\text{g/ml}$ unlabeled Fn. Same settings were used to generate flow conditions for 10 min at 500 s^{-1} (A) and 5000 s^{-1} (B) as well as to detect the amount of Fn fibrils assembled on adherent platelets. Values represent the mean \pm SD of two experiments (A) and three experiments (B). (■) control (▣) Reopro (■) LM609, ND: no detection

4. Discussion

Decades of research have shown several data to suspect Fn as one of the important players in platelet adhesion and aggregation [1, 33, 49-50, 54, 57-58, 68-71]. However, some of the data are inconsistent. Therefore, to understand more detail about the nature of its role in these processes, there are some challenging questions needed to be answered. (1) What is the precise role of Fn in hemostasis and thrombosis? (2) Is the conformational changes and assembly of Fn related to its role? (3) If so, which platelet receptors and co-factors are involved in these processes and are able to modulate its activity?

4.1 Purification of plasma Fn

To address those questions, Fn has to be first isolated in a highly purified intact form. There are several sources to obtain Fn. The relative high content of soluble Fn makes plasma the most convenient source for isolating this protein [72]. Due to the ability to bind to several molecules, it is quite difficult to obtain Fn with high purity. A lot of methods have been reported for the purification of plasma Fn but Fn obtained seemed to have low purity and yield. The most commonly used approach at present is gelatin affinity chromatography to purify Fn. In vitro, Fn can bind to gelatin with high affinity and this interaction is strongly dependent on pH and ionic strength [73]. Therefore, by using different composition of buffers for loading, washing and eluting, a desirable purity and amount of Fn can be achieved from plasma. Recently, a two-step purification methods combines two affinity chromatographies gelatin - arg or gelatin - heparin were described as strategy to obtain adequate purity with relative high yield of plasma Fn. However, these methods seem to be complex, time consuming and the use of Arg is expensive. In the present study, a simple, easy and inexpensive gelatin affinity chromatography was used for Fn purification. After thawing, plasma is added with 10 μ M EDTA which is the inhibitor for metal-dependent proteinases and 0.02 % NaN_3 to prevent the growth of bacteria. After loading of plasma containing Fn onto gelatin sepharose column, three washing steps are performed to eliminate non-specific binding proteins and Fg contamination. Fn was finally eluted with 3 M Urea buffer and immediately subjected to dialysis to remove urea to preserve structurally properties of the protein. Fn obtained in this method is homogenous as checked by SDS-PAGE in comparison with commercial Fn and Fg (Figure 3.1). By using this method, an acceptable

recovery of 50-60% of Fn from plasma can be achieved. The isolated Fn is biologically active because of its binding with activated platelets as seen by FACS analyses (supplemental data 3).

4.2 Dual role of Fn in platelet adhesion and aggregation

Regarding the question (1), Fn has been studied for the effect on platelet adhesion and aggregation. In literature, controversial reports have been published regarding to the potential role of Fn in platelet aggregation. In earlier studies, addition of plasma Fn had been shown to reduce platelet aggregation induced by thrombin, collagen, or ionophore A23187 [56-57, 71]. Moreover, a recent study using mice with a triple depletion of Fg/vWF/Fn showed that platelet aggregation and thrombus formation were enhanced in comparison with Fg/vWF double depleted mice [58]. However, two monoclonal antibodies against Fn (A3.3 and anti-Fn2) had been shown to reduce platelet aggregation. The monoclonal antibody A3.3 reduces the aggregation of platelets induced by thrombin or the ionophore A23187 while the anti-Fn2 antibody partially inhibits ADP or arachidonic acid stimulated platelet aggregation [71, 74-75]. To date, it remains unclear whether the negative effects of the two monoclonal antibodies might be caused by the recognition of functional epitopes on platelet surface molecules or on Fn secreted from platelet granules or of epitopes on plasma Fn.

In the attempt to study the effect of plasma Fn in platelet aggregation, platelet aggregation assays were performed with the addition of isolated plasma Fn. The results showed that Fn reduces platelet aggregation stimulated by PMA and collagen (Figure 3.2). This suppressive effect of Fn was also observed in the case of platelet aggregation induced by thrombin (supplement data 1).

The positive effect of plasma Fn on platelet adhesion has been demonstrated in several studies. Platelet adhesion onto collagen-coated surfaces was reduced when using Fn-free plasma but was restored when various concentrations of plasma Fn were added [50]. In addition, plasma Fn was shown to deposit in developing thrombi under high shear conditions using Fn-coated beads [54]. Therefore, the results presented here are consistent with these previous reports because they demonstrate an enhancement of platelet adhesion onto immobilized ligands by adding plasma Fn (Figure 3.3).

4.3 Functions of Fn in association with its unfolding and assembly

How can plasma Fn have two oppositional effects of promoting platelet adhesion but reducing platelet aggregation? The model of thrombus formation is well known to start with platelet adhesion on wounded site, followed by platelet activation supported by the actions of vWF and Fg molecules to recruit more platelets and form stable thrombi. The same model has been described for the activity of Fn in the processes of hemostasis where adherent platelets assemble Fn into fibrils so that those fibrils potentially connect platelets-molecules and platelets-platelets to enhance and stabilize thrombus formation (Figure 4.1). To support this model, several reports demonstrated the detection of Fn fibrils on adherent platelets to some specific thrombogenic surfaces and the enhancement of thrombogenicity of platelets in the presence of exogenous Fn under flow conditions. However, plasma Fn in solution folds into a compact structure that is unreactive to other molecules and does not undergo fibril formation [76-79]. Therefore, Fn has to become unfolded by binding to integrins to promote the assembly process. The ability of integrins to unfold Fn is tightly regulated by outside-in and inside-out signaling which regulates conformational changes of integrins and the organization of the actin cytoskeleton [20, 80-83]. It has been observed that suspended platelets and adherent platelets exhibit differences in the signaling pathway when interacting with Fg as well as being activated by agonists (data not shown). Based on this observation, it had been postulated that adherent platelets but not suspended platelets are able to unfold Fn and assemble Fn into an active fibrillar matrix. This may explain why Fn has a supportive effect in platelet adhesion but has a suppressive effect on platelet aggregation. There are several evidences that support this hypothesis. First, to our knowledge, although Fn was shown to interact with activated platelets in suspension, there is no study reporting the assembly of Fn on these platelets [66]. Second, Fn does not bind to ADP-stimulated platelets in suspension but interacts and assembles on ADP-stimulated adherent platelets [66, 84]. Third, adherent platelets demonstrate differences in intracellular signaling (supplemental data 4) that potentially lead to differences in integrin activation and ligand binding activities in comparison with suspended platelets.

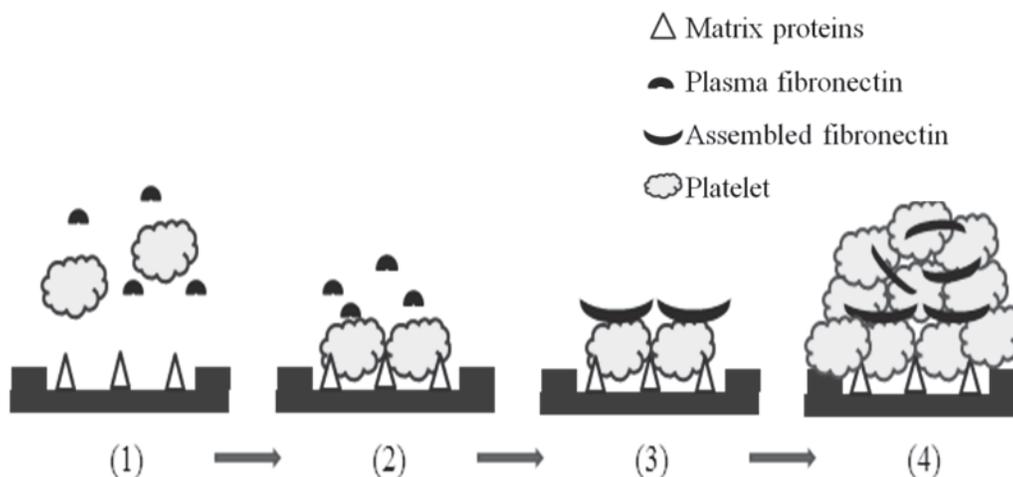


Figure 4.1: Putative model for role of fibronectin in platelet adhesion and aggregation

(1) Disruption of endothelium of a blood vessel causing the exposure of matrix proteins such as collagen, vWF, or Fn. (2) Circulating platelets adhere to the injury area via interactions with matrix proteins and thus become activated. (3) Activation of platelets leads to the interaction of integrins on platelet surface with plasma Fn causing the unfolding and fibril assembly of Fn. (4) Assembled Fn enhance molecules platelet aggregation by cross-linking adjacent platelets to stabilize platelet aggregates.

To confirm the hypothesis, a suitable tool to observe Fn unfolding *in vitro* has to be established first. FRET has been successfully used in previous studies to observe changes in the conformation of Fn in the extracellular matrix as well as after absorbing to different surfaces [24, 85]. By applying FRET-labeled Fn in this study, I could show that adherent platelets but not suspended platelets progressively unfold Fn during interaction (Figure 3.5). There are two models of conformational changes of Fn that can be detected by FRET measurement: compact-to-extended conformation (arm separation) without loss of secondary structure or compact-to-unfolded conformation change [24]. Due to the experimental settings, the obtained data of about 6 % decrease in FRET signals in the present study does not give any direct information about the exact conformational changes of Fn in this case. However, based on studies reporting the detection of Fn fibrils on adherent platelets, it can be noted that the observed conformational changes is not simply a process of opening Fn's arms. Indeed, it is a compact-to-extended process in Fn's modules which is required for Fn fibril formation. It has been reported that adherent platelets on different thrombogenic surfaces showed different abilities in assembling Fn fibrils on their surfaces [60]. Here I have shown that Fn assembly is not only dependent on the types of thrombogenic surface but also dependent on the adhesion of platelets.

In 2009, Reheman et. al. have reported that platelet aggregation and thrombus formation is enhanced in mice lacking Fg/vWF/Fn compared with Fg/vWF double deficient mice. Based on this data, the authors proposed that Fn is an inhibitor of thrombus formation in the absence of Fg/vWF and Fg and/or vWF are important for Fn to support platelet thrombus formation on thrombogenic surfaces [58]. In another study, fibrin was shown to cross-link and support Fn assembly in platelet thrombi to enhance thrombogenicity [53]. Together with the present observation that Fn unfolding by adherent platelets is enhanced in the presence of Fg at physiological concentrations (supplemental data 5), the inhibition of Fn on thrombus formation in double Fg/vWF deficient mice is likely because adherent platelets under these conditions can not induce Fn unfolding and assembly. Taken together, I conclude that Fn inhibits aggregation of platelets in suspension but supports platelet adhesion and thrombus formation onto thrombogenic surfaces. This oppositional effect can be explained by the fact that only adherent platelets can unfold and assemble Fn. Folded Fn may act as a competitor for Fg binding sites on suspended platelets while the unfolded form is a bridge to connect platelets-ligands as well as platelets-platelets to enhance adhesion and thrombus formation (Figure 4.2). In summary, these data provide strong answer for the question 2 that there is a relation between Fn unfolding and its activities in hemostasis and thrombosis. Ideally, drugs targeting thrombotic diseases should not alter normal hemostasis [68]. The results of the present study could suggest that Fn is a potential target for designing a novel drug by controlling its unfolding and assembly process although additional experiments are needed to be done.

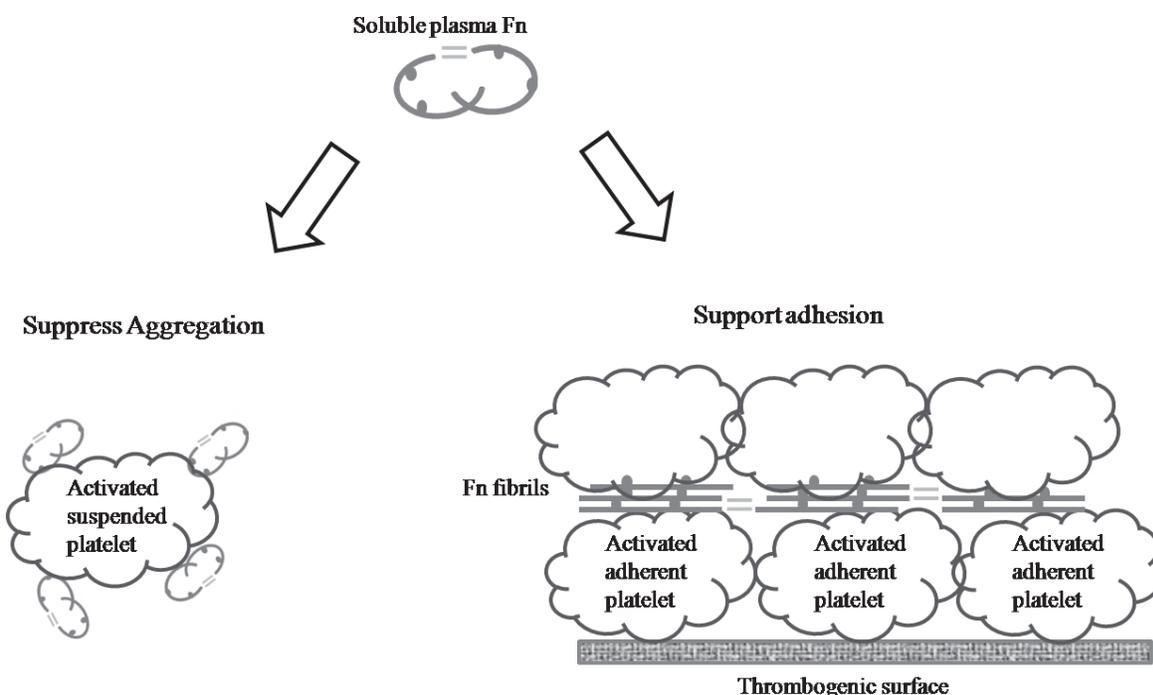


Figure 4.2: Depiction of the effect of Fn unfolding in platelet adhesion and aggregation

Plasma Fn is a compact dimeric protein in which the cryptic binding sites for self-assembly and interaction with other molecules are inaccessible. Upon binding to activated adherent platelets, Fn becomes unfolding, exposes the cryptic binding sites leads to the formation of active fibrils on platelet surface and subsequently enhances platelet-platelet interaction. However, when binding to activated suspended platelets, Fn can not be unfolded to form the active fibrils. Binding of the compact Fn to suspended platelet acts like a competitor with other molecules which are important for platelet aggregation such as Fg and therefore, platelet aggregation is suppressed.

4.4 Factors affect unfolding of Fn by adherent platelets

As having confirmed the relation between Fn unfolding and its role in hemostasis, it is important to know which factors can affect the conformational changes of Fn on adherent platelets. Elaborated studies on Fn assembly by fibroblast have demonstrated the importance of integrin types, mechanical stress induced by flow, integrin signaling, and cytoskeleton reorganization in regulating the process [20, 80-83, 86-91]. Fn assembly by platelets is suggested to have similar if not identical mechanisms as fibroblast [92]. Therefore, regarding the question 3, I examined the effects of integrins, cytoskeleton reorganization and shear stress conditions on Fn unfolding by adherent platelets.

4.4.1 $\beta 3$ integrin-dependent Fn unfolding by adherent platelets under static condition

Platelet expresses several integrins including three members of $\beta 1$ integrins ($\alpha 2\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$) and both members of $\beta 3$ integrins ($\alpha IIb\beta 3$ and $\alpha v\beta 3$) [32]. As $\alpha IIb\beta 3$ is the major integrin receptor on platelet which is known to play an indispensable role in platelet adhesion and

aggregation [63], it is critical to start with this integrin. Moreover, it is also worth to study the role of $\alpha v\beta 3$ because it shares the same $\beta 3$ subunit and 50 % homology in the α subunit with $\alpha IIb\beta 3$ [36]. Indeed, the $\beta 3$ integrins on platelets are reported to be involved in Fn fibril assembly under static and flow conditions [33]. Fn is thought to initially interact with the cell surface receptor via the 70 kDa N-terminal domain. The receptor for this initiation process has not yet identified. However, it has been suggested that $\alpha v\beta 3$ is involved in this process [44, 93]. It is well established that progression of Fn fibril assembly requires the binding of RGD motif to cell surface integrins [1]. Several integrins were reported to bind with RGD loop of Fn including $\alpha v\beta 3$ and platelet specific $\alpha IIb\beta 3$ [94]. These information can be used to interpret my findings showing the contribution of $\beta 3$ integrins in unfolding Fn on the platelet surface. Under static conditions, the fact that Reopro can completely block the unfolding of Fn by adherent platelets suggesting that $\beta 3$ integrins play a crucial role in this case. However, LM609 can block about half of the maximal FRET decrease in experiments without antibody indicating that $\alpha v\beta 3$ share the same contribution in Fn unfolding compared to $\alpha IIb\beta 3$ (Figure 6). This is a very interesting finding because there are only small numbers of 50-100 $\alpha v\beta 3$ receptor copies compared with 80,000-100,000 $\alpha IIb\beta 3$ receptor copies per platelet [32]. This finding implicates the importance of $\alpha v\beta 3$ despite of its low expression on platelets. Since platelets also expresses $\alpha 5\beta 1$ which is well characterized as Fn receptor [3, 32], further studies should focus on this integrin to understand more about the Fn unfolding process by adherent platelet.

4.4.2 Fn unfolding by adherent platelet can be modulated by cytoskeleton drugs

Interaction between fibronectin and the activated cellular integrins is not sufficient for Fn unfolding and assembly. Cells with truncated β cytoplasmic tails, or treated with drugs that inhibit actin polymerization are unable to assemble Fn [20, 82, 95]. Those reports implicate the importance of subsequent events of ligand binding for Fn unfolding and assembly. As dimer molecules, binding of Fn to integrins induces receptor clustering, integrin intracellular signaling that lead to the activation of actin polymerization [3]. The polymerization of actin filaments generates crucial tensions and forces that are transmitted across integrins to induce conformational changes of Fn [3]. The fact that CytoD and Lat A inhibit Fn unfolding by adherent platelets in a concentration-dependent manner is in consistence with those previous reports. In contrast, enhancing actin polymerization by Jas leads to the acceleration of Fn unfolding process by adherent platelets. These data strongly indicate that Fn unfolding process

can be controlled through modulation of actin polymerization and hence, cellular tension and forces. The affinity of integrins to Fn are not changed when cells are pre-treated with CytoD [20]. Therefore, the present observation of changes in Fn unfolding kinetic by adherent platelets treated with cytoskeletal drugs are only because of the changes in tension forces generated by actin polymerization.

4.4.3 Acceleration of Fn assembly by adherent platelets by shear stress under flow

The requirement for platelets to fulfill their role in hemostasis is that they can irreversibly attach to the site of injury that is strongly influenced by shear stress generated by blood flow [30]. Platelet responses to shear stress by mechanotransduction are mediated by integrins that lead to the reorganization of cytoskeleton by actin polymerization [48]. The observation that Fn unfolding kinetic can be modulated by cytoskeletal drugs suggests that flow is also one of the modulators for Fn unfolding. Studies on the assembly of Fn by fibroblast under static condition revealed that initial Fn fibrils are thin and DOC-soluble but then grow in length and thickness as well as become DOC-insoluble within 6 h [25]. Although affected by the excess of unlabeled Fn and the measurement area, my data which shows that FRET signals decrease slowly by 6 % within 3 h of incubation is likely consistent with the long time needed for Fn assembly under static condition (Figure 3.5). The finding that, under flow conditions, especially at a high shear rate of 5000 s^{-1} , DOC-insoluble fibrils can be detected even after 2 min of shear stress indicates that Fn assembly is strongly dependent on flow conditions. Platelet adhesion on Fn is less efficient than on collagen or vWF but is independent on shear rate over the range of $650\text{-}3400 \text{ s}^{-1}$ [51]. In addition, adherent platelets on Fn do not assemble Fn and hence platelet aggregation and thrombus formation are not enhanced under shear stress of 300 or 1250 s^{-1} [33]. In contrast to those reports, my results show that less platelets adhere on Fn surfaces (data not shown) when shear rates increase from 500 s^{-1} to 5000 s^{-1} but more DOC-insoluble Fn fibrils can be detected (Figure 3.8). The possible explanations for these contradictory results are (1) the present experimental setting uses a higher shear rate than that of those reports which can be over the mechanical threshold for Fn unfolding and assembly; (2) although DOC-insoluble fibrils can be detected under low shear rate of 500 s^{-1} , the amounts appear to be very small. Moreover, we can not exclude the possibility that Fn surfaces can interact with Fn molecules in solution, acting as a tether point for shear rate itself to induce Fn unfolding and then assembly. Indeed, shear stress has been reported to induce conformational changes in vWF [96]. Though further experiments

need to be done, based on our results, we can initially conclude that flow condition is an important factor for regulating Fn assembly.

4.4.4 Role of β 3 integrins (α III** β 3 and α v β 3) under flow conditions**

Under static condition, α I**II** β 3 and α v β 3 are observed to contribute equally to unfold Fn on adherent platelets (Figure 3.6). Since different integrins may respond differently to shear stress, it is worth to study their roles in Fn unfolding under flow conditions. The fact that platelets blocked with LM609 and Reopro show the same amount of detected Fn fibrils after 2 min exposure to high shear rate indicate that α v β 3 is more important in initiating the process. However, when platelets are exposed to the same high shear rate with increasing time, Reopro blocks more significantly the Fn fibril assembly on adherent platelets than LM609 does. The data implies that α I**II** β 3 is more important in the later phase of the progression. Taken together, despite of its lower expression on the platelet surface, α v β 3 shows an equivalent or even more contribution in initiating the Fn unfolding process under static or high flow conditions, respectively, as compared with α I**II** β 3. In contrast, α I**II** β 3 with its huge amount on platelet surface acts more essentially in the later phase to progress the process. The present data strongly supports the idea that not a single integrin or Fn binding domain, but many different molecules and Fn domains may be involved in unfolding and assembly of Fn [3].

5. Conclusions and perspectives

My studies focus on the conformational changes and assembly of Fn during interacting with platelets in hemostasis. By using FRET as a tool to observe Fn unfolding *in vitro*, I demonstrated that the molecular status of Fn can be used to explain its dual role in platelet adhesion and aggregation. Adherent platelets can unfold and hence, induce Fn fibril assembly to enhance adhesion whereas Fn is not unfolded by suspended platelets and therefore, inhibits platelet aggregation. My subsequent studies demonstrated the importance of $\beta 3$ integrins, actin polymerization, and shear stress induced by high flow conditions for adherent platelets to unfold and assemble Fn. I found that under static condition, the unfolding of Fn by adherent platelet is mainly mediated by $\beta 3$ integrins. Despite of its low expression in comparison with $\alpha \text{IIb}\beta 3$, $\alpha \nu \beta 3$ likely shows an equal contribution to unfold Fn. On the other hand, under flow conditions, the contributions of $\beta 3$ integrins are differ. $\alpha \nu \beta 3$ plays a more important role in the initial phase of Fn assembly under high shear rates whereas the role of $\alpha \text{IIb}\beta 3$ is more dominant in the later phase of progression. In addition, my data support the hypothesis that more than one type of integrins play a role in Fn unfolding and assembly. By applying cytoskeletal drugs to regulate the actin polymerization as well as the application of flow to generate shear stress, my data identified actin polymerization and shear stress as modulators for Fn unfolding and assembly on adherent platelets. Taken together, I found a relationship between Fn molecular status and its role in platelet adhesion and aggregation. Moreover, the process of Fn unfolding and assembly by adherent platelets is dependent on the type of integrin, actin polymerization and shear stress.

The insights of my data raise some new questions for further studies: 1. What are the mechanisms for the differences in the ability to unfold Fn between adherent and suspended platelets? 2. Since $\alpha 5\beta 1$ is known to be a primary receptor for Fn and might have a higher expression than $\alpha \nu \beta 3$, what is the role of this receptor in unfolding Fn on adherent platelets. 3. Whether or not flow itself can induce Fn unfolding and assembly? Such studies will give more insights into the process of Fn assembly by platelet and would better define the role of Fn in platelet adhesion and aggregation, and hence hemostasis and thrombosis.

Summary

My studies establish the relationship between conformational changes of Fn and its role in platelet adhesion and aggregation. Furthermore, I characterized the effect of $\beta 3$ integrins, actin polymerization, and shear stress on conformational changes of Fn on adherent platelets.

Fn was observed to play a dual role in hemostasis by decreasing platelet aggregation but enhancing platelet adhesion. Then, FRET was used as a tool to monitor Fn unfolding when interacting with adherent and suspended platelets *in vitro*. The presented data showed that adherent but not suspended platelets are able to unfold Fn during interaction. These observations lead to the conclusion that there is a relationship between Fn conformational changes and its role in hemostasis. Fn is unfolded by adherent platelets to support platelet adhesion whereas its conformation remains unchanged by suspended platelets and hence, inhibits platelet aggregation by acting like a competitor with important molecules such as Fg.

Experiments with cytoskeletal drugs and different flow conditions revealed that Fn unfolding and assembly are strongly influenced by actin polymerization and shear stress. Enhancing actin polymerization or increasing flow condition leads to the acceleration of Fn unfolding and assembly.

In addition, my results showed that there are different contributions of $\beta 3$ integrins in the processes of unfolding and assembling Fn on adherent platelet under static and flow conditions. Under static condition, $\alpha v\beta 3$ was observed to have an equal contribution as $\alpha I Ib\beta 3$ to Fn unfolding despite of its very low expression on the platelet surface. However, under high flow condition, the here presented data showed that $\alpha v\beta 3$ plays a more important role in initiating the Fn assembly process whereas $\alpha I Ib\beta 3$ has a more dominant role in the later phase of progressing. Moreover, my data supported the hypothesis that more than one type of integrins are required for Fn unfolding and assembly on adherent platelet.

Zusammenfassung

Die Beziehung zwischen der Konformationsänderung des Fibronektins und seiner Funktion bzw. Funktionsänderung während der Plättchenadhäsion und –aggregation stand im Fokus dieser Arbeit. Um diese Konformations-Funktions-Beziehung eingehend zu untersuchen, wurden die Beteiligung der $\beta 3$ -Integrine, die Aktinpolymerisation und der Scherstress als Steuerelemente der Fibronektinkonformation an adhärenen Plättchen im Detail analysiert.

In dieser Arbeit wird gezeigt, dass Fibronektin eine duale Rolle in der Hämostase spielen kann. Auf der einen Seite setzt Fibronektin die Plättchenaggregation herab, während es auf der anderen Seite die Plättchenadhäsion fördert. Dazu wurde zusätzlich die Entfaltung von Fibronektin auf der Oberfläche von adhärenen Plättchen und Plättchen in Suspension *in vitro* mittels Fluoreszenz-Resonanz-Energie-Transfer (FRET) untersucht. Die Ergebnisse belegen, dass adhärenen, aber nicht Plättchen in Suspension Fibronektin entfalten können. Diese Beobachtung legt die Schlussfolgerung nahe, dass es eine Wechselbeziehung zwischen Änderungen der Fibronektinkonformation und Hämostase gibt. Fibronektin wird auf adhärenen Plättchen entfaltet und fördert die Plättchenadhäsion, während es auf Plättchen in Suspension keine Konformationsänderungen zeigt. Daher kann Fibronektin als ein Gegenspieler von Fibrinogen wirken und die Plättchenaggregation inhibieren.

Zytoskelettgifte oder flusssdynamische Bedingungen haben gezeigt, dass die Entfaltung von Fibronektin und seine Aggregatbildung stark von der Polymerisation des Aktinszytoskeletts und vom Scherstress beeinflusst werden. Eine gesteigerte Aktinpolymerisation oder zunehmender biomechanischer Stress beschleunigen die Entfaltung und Aggregatbildung von Fibronektin.

Zusätzlich belegen die Ergebnisse, dass die $\beta 3$ -Integrin unter statischen und flusssdynamischen Bedingungen unterschiedlich an der Fibronektinentfaltung und Aggregatbildung auf adhärenen Plättchen beteiligt sind. Unter statischen Bedingungen sind $\alpha v\beta 3$ und $\alpha IIb\beta 3$ gleichermaßen an der Entfaltung von Fibronektin beteiligt, dies, obwohl $\alpha v\beta 3$ weniger auf der Plättchenoberfläche exprimiert wird als $\alpha IIb\beta 3$. Dennoch unter sehr hohen Flussgeschwindigkeiten das zeigen die hier vorgestellten Ergebnisse, übt $\alpha v\beta 3$ eine bedeutsamere Rolle bei der Initiierung der Aggregatbildung aus, während $\alpha IIb\beta 3$ eine dominante Rolle in der späten Phase der Aggregatbildung hat. Darüber hinaus unterstützen die Ergebnisse die Hypothese, dass mehr als

ein Integrin benötigt wird, damit Fibronectin auf Plättchen entfalten wird und sich Aggregate bilden können.

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Appendix

Binding of isolated Fn to platelets measured by FACS

5 μ l of platelet suspension (2.5×10^8 /ml) was diluted in 50 μ l PBS buffer pH 7.3 containing activation agonist (10 μ M ADP or 40 nM PMA) and 20 μ g/ml Alexa 488 conjugated Fn (Fn488). Samples were incubated for 30 min at room temperature followed by the addition of 500 μ l PBS pH 7.3 to stop the reaction and subjected to FACS analyses. In control experiments, FACS analyses were performed with samples of platelet alone or without agonist.

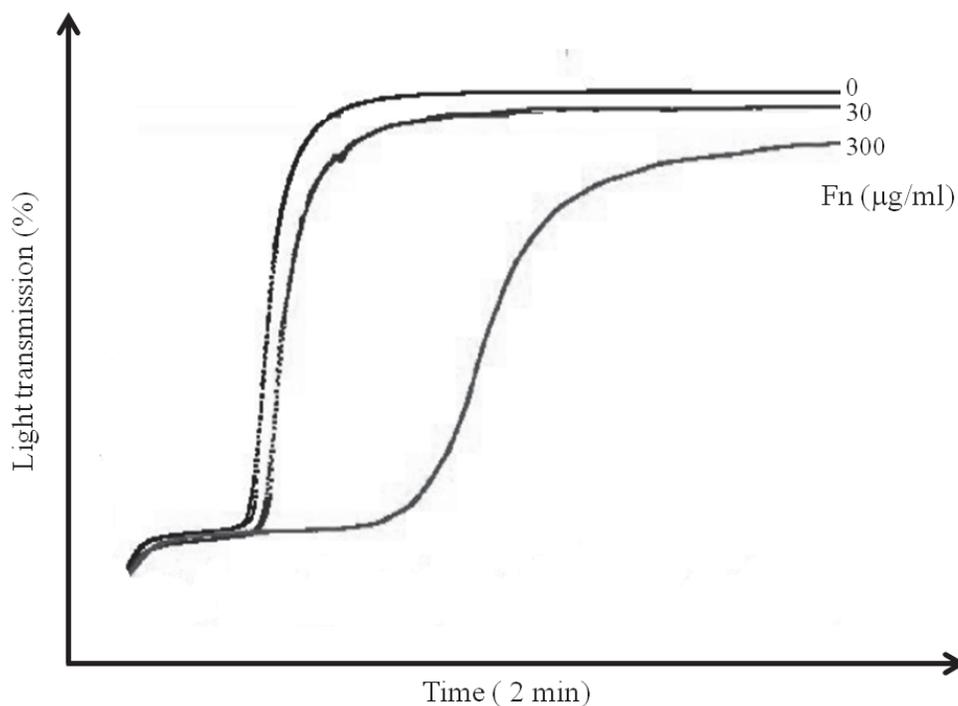
Relative quantification of platelet adherent to immobilized Fn

Washed platelets in suspension were adjusted to a concentration of 5×10^8 /ml in HEPES-Tyrode's buffer. A series of platelet counts from 5×10^5 to 10^7 were lysed with 200 μ l of lysis buffer (20 mM Tris, 1 % Triton X-100, 0.5 % Na deoxycholate, 5 mM EDTA, 145 mM NaCl, 1 mM Na_3VO_4 , 0.1 mM NaF, protease inhibitor cocktails (Roche), pH 7.4). The lysates were incubated for 1 hour on ice and the protein contents were determined by using Bio-Rad protein assay kit (Bio-Rad). The standard curve between platelet count and total protein concentration was plotted using Microsoft Excel. In another experiments, 2 ml of 10^8 /ml washed platelets in suspension were allowed to adhere to the surface of PMMA cuvettes precoated with 50 μ g/ml Fn for 1 – 3 h at 37 °C in the presence or absence of Reopro (10 μ g/ml) or LM609 (5 μ g/ml). After indicated incubation times, unbound platelets were removed by three times washing with PBS pH 7.3. Adherent platelets were lysed with 200 μ l lysis buffer. Samples were subjected to protein assay to compare the amount of adherent platelets.

Tyrosine phosphorylation of Src pY416

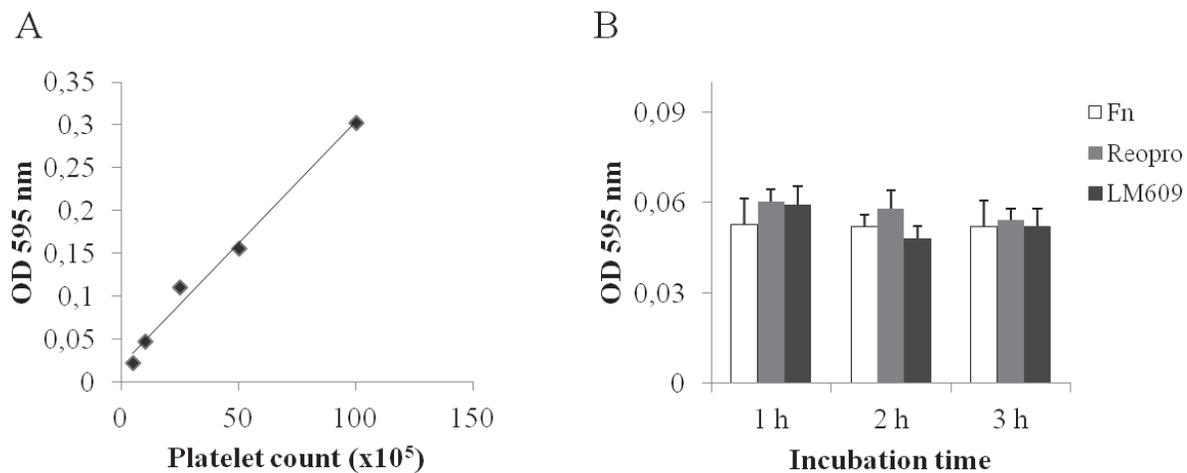
The concentration of washed platelets was adjusted to 5×10^8 /ml by using HEPES-Tyrode's buffer with 2 mM CaCl_2 added prior to the experiments. For experiments with adherent platelets, 150 μ l of washed platelets were allowed to adhere to wells coated with 10 μ g/ml fibronectin or 0.5 % heat inactivated bovine serum albumin (control experiments) for 15 minutes at 37°C. In parallel experiments, 10 μ M ADP, 40 nM PMA or 0.5 mM MnCl_2 was added to washed platelets prior to adhesion. For experiments with suspended platelets, 150 μ l of washed platelets in suspension were incubated with 300 μ g/ml fibronectin for 15 minutes at 37°C in the absence or presence of agonists (10 μ M ADP, 40 nM PMA or 0.5 mM MnCl_2). Adherent platelets and

suspended platelets were collected after incubation. In both instances, platelets were lysed with ice-cold lysis buffer. The lysates were incubated for 1 hour on ice and the protein contents were determined by protein assay. Lysates containing equal amounts of protein (60 μg) were applied on a 6 % SDS-PAGE gel, transferred to PVDF membranes, and probed with anti-pp416^{Src} antibody (Invitrogen) followed by anti-rabbit antibody (Amersham) and developed as described by the user's manual. The signals were scanned with Bio-Rad VersaDoc imaging system and the densitometric quantitation was performed by using Quantity One software (Bio-Rad).



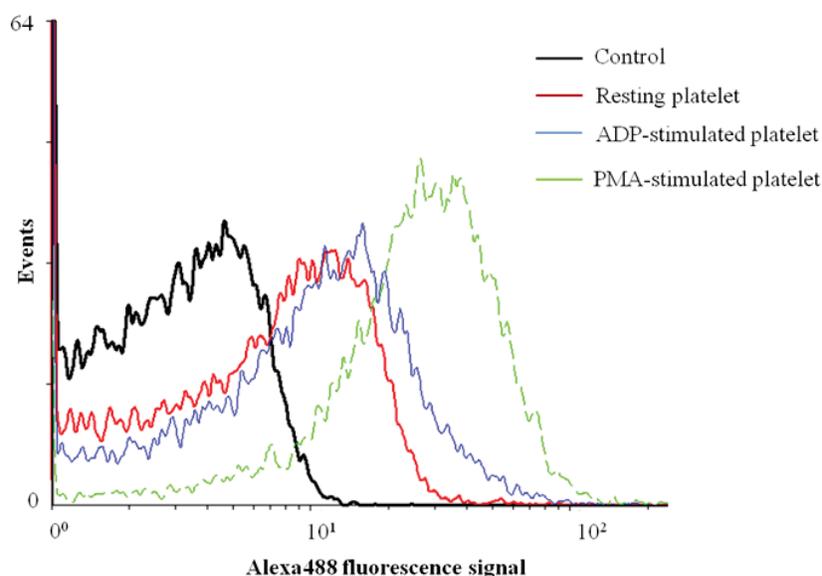
Supplemental data 1: Fx decreases platelet aggregation induced by thrombin in a concentration dependent manner

Washed platelets were stimulated with 1 U/ml thrombin in the presence or absence of 30 $\mu\text{g/ml}$, or 300 $\mu\text{g/ml}$ plasma Fx.



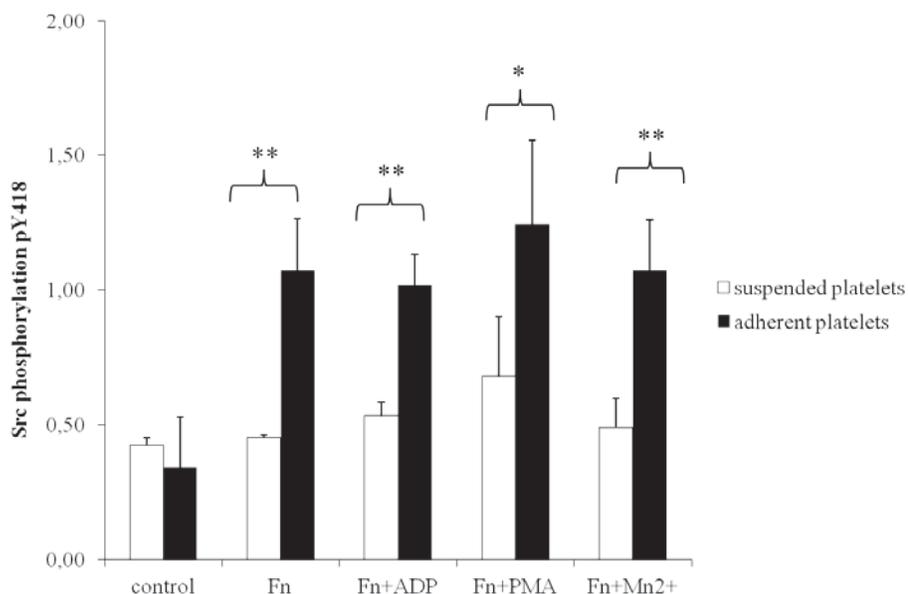
Supplemental data 2: Effect of β 3 integrin-blocking antibodies on the relative quantification of platelet adherent on immobilized Fn.

- (A) Calibration curve of known platelet counts and OD 595 nm of total protein derived from lysates. (B) Comparison of total proteins derived from platelet adherent to immobilized Fn with or without treatment of Reopro or LM609 after 1-3 h of incubation. Data obtained from Dr. Marianna Gyenes.



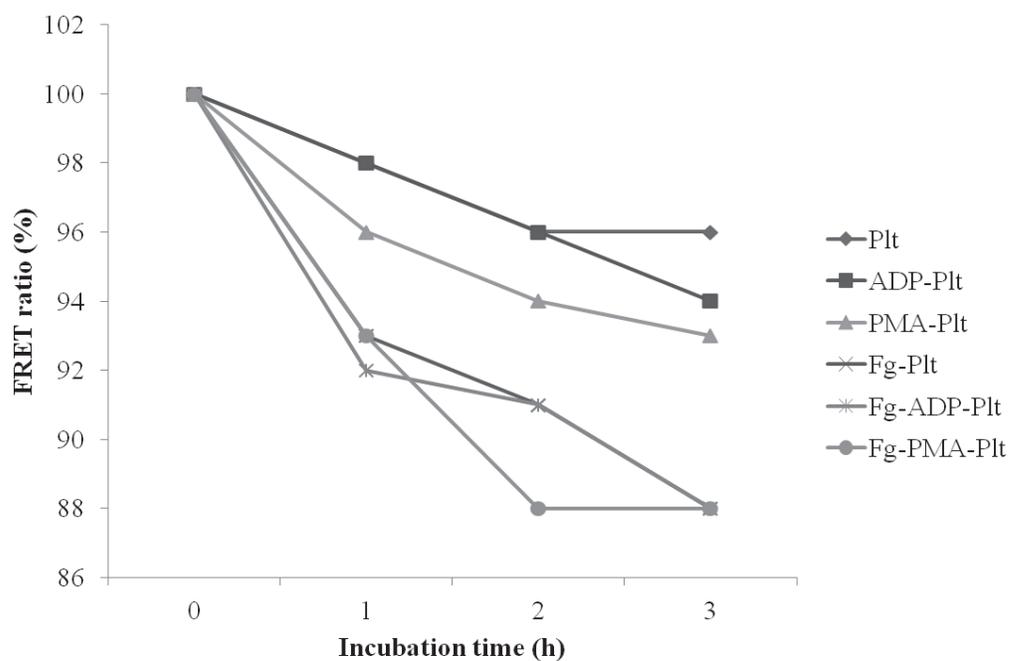
Supplemental data 3: FACS analysis of isolated Fn binding to platelets

Isolated Fn was conjugated with Alexa488 and subjected to FACS analysis for binding to resting platelet, ADP-activated platelet and PMA-activated platelet. For control, signals from platelets without Alexa488-Fn were also recorded.



Supplemental data 4: Src phosphorylation analysis of adherent and suspended platelets after interacting with Fn

For experiments with adherent platelets, washed platelets were allowed to adhere on immobilized Fn or 0.5 % BSA (control experiments) for 30 min at 37°C. In parallel experiments, 10 μ M ADP or 40 nM PMA or 0.5 mM MnCl₂ were added to platelets after adhesion for 15 min. For experiments with suspended platelets, washed platelets were incubated with Fn for 15 min at 37°C in the absence or presence of agonists (10 μ M ADP or 40 nM PMA or 0.5 mM MnCl₂). Both types of platelets were collected and lysed (see Materials and Methods). Lysates containing equal amounts of protein were subjected to Western blot probed with anti-ppSrcY416 antibody. The signals were scanned with Bio-Rad VersaDoc imaging system and densitometric quantitation was done by using Quantity One software (Bio-Rad). Values represent the mean \pm SD of three experiments. (*) p=0.06, (**) p<0.01. Data obtained from Dr. Marianna Gyenes.



Supplemental data 5: FRET measurements of Fn unfolding by adherent platelets in the presence of agonists or/and Fg

10 $\mu\text{g/ml}$ Fn mixtures were incubated with platelets adherent to immobilized Fn (50 $\mu\text{g/ml}$) with or without 10 μM ADP, or 40 nM PMA, or 3 mg/ml Fg for 3 h at room temperature