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Fibronectin Is Required for Platelet Adhesion and for Thrombus Formation on Subendothelium and Collagen Surfaces

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Fibronectin (FN) plays a role in several adhesion mediated functions including the interaction of platelets with subendothelium. We investigated the role of plasma FN in platelet adhesion and platelet thrombus formation under flow conditions. We used two different perfusion models: the annular chamber with α-chymotrypsin–treated rabbit vessel segments, and the flat chamber with coverslips coated with fibrillar purified human collagen type III. Perfusates consisted of washed platelets and washed RBCs, suspended in normal or FN-depleted plasma. Perfusions were carried out for ten minutes at shear rates of 300 or 1,300 s^{-1}. Platelet deposition and thrombus dimensions were evaluated morphometrically by a computerized system. We found that depletion of plasma fibronectin significantly reduced the percentage of total coverage surface and percentage of platelet thrombus, at both shear rates studied, and in both perfusion systems ($P < .01$) ($P < .01$). The dimensions of the platelet thrombi formed in perfusions at high shear rate were also significantly reduced in perfusions carried out with FN depleted plasma ($P < .01$). Addition of purified FN to FN-depleted perfusates restored all values to those measured in the control perfusions. These results indicate that plasma FN is required for platelet aggregate and thrombus formation following adhesion under flow conditions.

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PLATELET ATTACHMENT, spreading and aggregation on vascular subendothelium are crucial events in platelet thrombus formation. Several factors are known to regulate these events: physical factors,[1,2] subendothelial components,[3] adhesive proteins,[4] and specific receptors on the platelet membrane.[5]

Fibronectin (FN) is a multifunctional glycoprotein with numerous biological roles. Besides its interactions with platelets, FN is known to promote attachment and spreading of cells on collagenous substrates,[6] and to bind to different types of cells such as fibroblasts,[7] rat kidney cells,[8] and malignant melanoma cells.[9] Recent studies have demonstrated that the Arg-Gly-Asp sequence within FN is involved in the cell attachment function of this glycoprotein. It has been hypothesized that platelets have a receptor capable of recognizing the Arg-Gly-Asp sequence in several adhesive proteins and that this platelet receptor is the membrane glycoprotein GPIIb/IIIa.[10] This amino acid sequence has also been found in other proteins that interact with platelets such as fibrinogen, von Willebrand factor, and vitronectin.[11] On the other hand, it has been reported that platelets from thrombasthenic patients lacking the GPIIb/IIIa complex, do not form thrombi in the Baumgartner perfusion system, using both citrated[12] and nonanticoagulated blood.[13]

In this study, we investigated the role of FN in platelet adhesion and in platelet thrombus formation. We used two perfusion models: the annular chamber with rabbit abdominal aorta segments and the flat chamber with coverslips coated with fibrillar purified human collagen type III. Morphometry was used to measure the extent and the state of interacting platelets, as well as to determine platelet thrombus dimensions.

MATERIALS AND METHODS

Collagens

Collagen type III from human umbilical arteries was isolated and purified by the Chandrajajan method.[14] Collagen fibers were prepared as previously described.[15] Briefly, native type fibers were prepared by dissolving the collagen (1 mg/mL) in 50 mmol/L acetic acid and dialized against 20 mmol/L Na_2HPO_4 at 4°C for 48 hours. The collagen fibrils were collected by centrifugation (30 minutes, 30,000 g, 4°C) and resuspended in 20 mmol/L Na_2HPO_4. The collagen concentration in the fibrillar suspension was determined by the hydroxyproline assay.

Fibronectin Depleted Plasma and Fibronectin

FN was isolated from plasma by affinity chromatography as described.[16] FN-depleted plasma contained no detectable FN on electro-immunoassay. The level of FVIIIR:Ag was unchanged and the level of FVIII:RCo ranged from 0.7 to 0.85 U/mL. There was essentially no change in the multimeric structure of von Willebrand factor after passage over the gelatin column. Purity of the isolated FN was assessed by SDS gel electrophoresis according to Laemmli.[17]

Preparation of Perfusates

Blood from normal healthy donors who had taken no drugs ten days before blood collection was anticoagulated with 1:10 vol 110 mmol/L trisodium citrate. Platelet rich-plasma was prepared by centrifugation (ten minutes, 150 g, 20°C). Platelets were washed twice by centrifugation (ten minutes, 500 g, 20°C) in Krebs-Ringer buffer pH 6.0. RBCs were washed three times by centrifugation (ten minutes, 300 g, 20°C) in isotonic saline containing 5 mmol/L glucose. Perfusates were prepared as previously described[15] and consisted of washed platelets (WP), washed RBCs (WRBC) and normal plasma or FN-depleted plasma. Before each perfusion run, the perfusate (15 mL) was reconstituted by adding WRBC to the resuspended platelets (hematocrit 0.4, platelet count 1.9×10^{11} pl/L, final concentration). In some experiments, FN was added to

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Table 1. Platelet Deposition on α-Chymotrypsin–Treated Rabbit Vessels

Perfusate	Vessel Surface Covered (%)				
	Contact	Spread	Ag < 5μm	Thrombus	Surface Coverage
Normal plasma	3.6 ± 1.0	6.8 ± 0.1	3.6 ± 0.3	3.5 ± 0.5	17.6 ± 0.5
FN-depleted plasma	2.7 ± 0.1	4.0 ± 0.3	1.5 ± 0.3*	1.5 ± 0.3*	9.8 ± 0.4*
FN-depleted plasma + FN	4.0 ± 2.0	6.6 ± 0.2	3.2 ± 0.9	3.0 ± 1.0	16.8 ± 0.9

α-Chymotrypsin–digested rabbit vessels exposed to blood for ten minutes at a shear rate of 300 s^{-1}. Values represent mean ± SEM (n = 5).
*$P < .01$.

FN-depleted plasma to obtain a final concentration of 300 μg/mL. The perfusates were incubated for five minutes in a water bath at 37°C before starting the perfusion.

Preparation of the Vessels

Rabbit abdominal aorta segments were obtained from New Zealand rabbits that weighed 2.5 to 2.8 kg. Arteries were excised above the iliac artery and were everted to expose the luminal surface. The endothelium was removed by treatment with α-chymotrypsin as previously described.[18] The everted, de-endothelialized segments, ~14 mm in length, were stored at −80°C until used. Before perfusion, vessel segments were rinsed with 0.02 mol/L phosphate buffered saline (PBS) pH 7.4.

In some experiments, α-chymotrypsin–digested rabbit segments were incubated with purified FN at a concentration of 1 mg/mL for 30 minutes at 20°C. The preincubated vessel segments were thoroughly rinsed with PBS pH 7.4 before being used in perfusions.

Preparation of the Coverslips

Fibrillar collagen (1 mg/mL) was sprayed onto plastic coverslips (Thermanox, Miles Laboratories, Naperville, IL) at a concentration of 20 μg/cm² with a retouching airbrush, as previously described.[15]

Perfusion Experiments

Perfusions were carried out in annular chambers according to Baumgartner,[19] and in flat chambers as described by Sakariassen et al.[20] The annular chambers were used for the arteries and the flat chambers were used for collagen-coated coverslips.

Processing of Vessel Segments and Plastic Coverslips for Cross-Sectional Morphometric Evaluation

Vessel segments. After perfusion, the segments were rinsed with PBS and fixed with glutaraldehyde. The fixed segments were sliced from the supporting rod, dehydrated through a graded series of ethanol concentrations, embedded in JB-4 embedding media (KIT composed of components A, B, and C Polysciences, Warrington, PA), thin-sectioned for light microscopy, and stained with toluidine blue as described.[21]

Collagen-coated coverslips. In perfusions carried out with the flat chambers, the system was rinsed with PBS pH 7.4. following the experiments. The perfused coverslips were carefully removed from the chamber knobs, rinsed thoroughly with 0.02 mol/L PBS pH 7.4 and fixed for one hour in 0.5% glutaraldehyde in 0.1 mmol/L PBS pH 7.4. After one hour of fixation, coverslips were washed in fresh PBS and dehydrated in a graded series of ethanols (30%, 50%, 70%, 96%). Infiltration of the samples was performed in catalyzed JB-4 solution for two hours at 4°C. Embedding of the coverslips was performed in flat molds. The nonperfused side of the coverslips was laid on the bottom of the mold filled with catalyzed JB-4 solution. Polymerization was carried out at 4°C in special containers from which air had been removed by nitrogen current. After complete polymerization, plastic coverslips were carefully peeled off from the JB-4 polymer, leaving the perfused side of the coverslip firmly embedded in the JB-4 polymer. Three-millimeter wide strips of the JB-4 flat blocks were appropriately oriented and vertically embedded in BEEM capsules as described above. Final blocks were vertically sectioned with glass knives. Sections were removed from the cutting edge, floated on water, mounted on glass slides, dried, and stained with 1% methylene blue.

Morphometric Evaluation

Platelet interaction with subendothelium was evaluated according to the basic criteria described by Baumgartner[19] with minor modifications.[21] Basically, platelets or groups of platelets were classified as follows: contact (C), platelets that are attached but not spread on the subendothelium; spread (S), platelets that have spread on the subendothelial surface; aggregate (Ag), aggregates of <5 μm in height; thrombus (T), platelet aggregates of ≥5 μm in height. Total covered surface was obtained by adding C + S + Ag + T. Morphometric evaluation was performed using a manual optical picture analysis system (MOP 20 Kontron) connected to a computer with an automated recognition program, as previously described.[21]

In addition to the morphometric evaluation, we simultaneously determined the cross-sectional areas of all platelet aggregates of >5 μm in height (Thrombus). The sum of all thrombus surface area expressed in square micrometers was related to the perimeter of either rabbit vessel or collagen coated coverslips, in each case 3,400 μm. The result obtained corresponded stereologically to the thrombus volume per unit of surface area of the subendothelium ($\mu m^3/\mu m^2$), as described elsewhere.[22]

Table 2. Platelet Deposition on α-Chymotrypsin–Treated Rabbit Vessels

Perfusate	Vessel Surface Covered (%)				
	Contact	Spread	Ag < 5μm	Thrombus	Surface Coverage
Normal plasma (n = 7)	3.9 ± 0.6	10.8 ± 0.4	3.3 ± 0.5	14.4 ± 1.0	29.2 ± 3.7
FN-depleted plasma (n = 6)	3.9 ± 0.4	8.9 ± 1.7	1.6 ± 0.5	1.6 ± 0.1*	14.7 ± 3.0*
FN-depleted plasma + FN (n = 3)	4.1 ± 0.9	13.2 ± 2.8	6.5 ± 2.2	8.6 ± 1.8	32.4 ± 6.3

α-Chymotrypsin–digested rabbit vessels exposed to blood for ten minutes at a shear rate of 1,300 s^1. Values represent mean ± SEM with the number of experiments in parentheses.
*$P < .01$.

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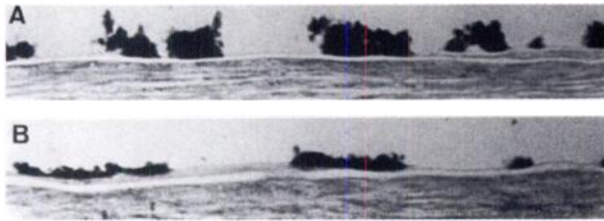

Fig 1. Light micrographs (original magnification ×1,000; current magnification ×590) of α-chymotrypsin treated rabbit subendothelium after ten minute perfusions at a shear rate of 1,300 s^{-1}. (A) Normal plasma. (B) FN-depleted plasma.

RESULTS

Studies on Platelet Deposition on Rabbit Subendothelium

To assess the role of plasma FN in the interaction of platelets with subendothelium of different thrombogenic properties, we carried out perfusions with vessel segments that had been de-endothelialized by overnight digestion with α-chymotrypsin. Perfusions were performed at two different shear rates, 300 and 1,300 s^{-1}, respectively. Table 1 summarizes the results obtained in the perfusions at a shear rate of 300 s^{-1}. In this group of experiments, plasma FN depletion significantly reduced the percentage of platelet thrombi from 3.5% ± 0.5% to 1.5% ± 0.3% ($P < .01$) and the total coverage surface 17.6% ± 0.5% to 9.8% ± 0.4% ($P < .01$). The percentage of the surface covered by contact and spread platelets, and the platelet aggregates of <5 μm in height were also reduced, although these values were not significant.

Perfusions carried out with α-chymotrypsin–digested rabbit vessels at a high shear rate (1,300 s^{-1}) indicated the role of plasma FN in platelet thrombus formation (Table 2). In these experiments, the surface coverage with platelet thrombi decreased from 14.4% ± 1.0% to 1.6% ± 0.1% ($P < .01$). The total surface coverage was also significantly diminished from 29.2% ± 3.7% to 14.7% ± 3.0% ($P < .01$). No significant differences were observed for the values of contact and spread platelets. Surface coverage with aggregates of <5 μm in height was slightly decreased but of no statistical significance. Figure 1 illustrates the differences by showing micrographs of the vessel wall subendothelium after perfusions at a shear rate of 1,300 s^{-1} with perfusates prepared with normal plasma (Fig 1A) or FN-depleted plasma (Fig 1B).

In both groups of experiments, at low and high shear rates, addition of 300 μg/mL of FN to FN-depleted perfusates restored the values of platelet deposition to those measured in control perfusions.

Treatment of the rabbit vessel with α-chymotrypsin for 18 hours digests all noncollagenous subendothelial proteins, including FN. Attempts to restore vessel FN were made by preincubating the digested vessels with purified human FN. Preincubation of enzymatically-treated rabbit vessel segments with purified human FN (1 mg/mL) for 30 minutes at 22°C did not modify the pattern of platelet adhesion. These results are summarized in Table 3. No significant changes were observed after preincubation of the vascular material with purified FN. For these experiments, FN-depleted plasma was used in the perfusate.

Platelet Deposition on Nonfibrillar and Fibrillar Purified Collagen Type III

To study the role of plasma FN on platelet adhesion to human purified collagen type III, perfusions using the flat chamber perfusion system were performed.[20]

Results are summarized in Table 4. At a shear rate of 300 s^{-1}, the vessel surface covered with platelet aggregates of <5 μm, with platelet thrombi, and the total coverage surface, were reduced from 14.2% ± 1.7% to 8.3% ± 0.8%, from 6.1% ± 1.7% to 2.0% ± 0.7%, and from 31.0% ± 5.0% to 14.8% ± 3.7%, respectively in perfusions carried out with FN-depleted plasma. All these values reached significant levels ($P < .01$).

The values obtained in perfusions performed at a shear rate of 1,300 s^{-1} also indicated the role of plasma FN in the interaction of platelets with purified fibrillar collagen type III. The most significant reduction observed in perfusates prepared with FN-depleted plasma was in the percentage of platelet thrombi, with a decrease from 14.3% ± 3.0% to 3.6% ± 1.0% ($P < .05$). The values of platelet aggregates and of the total coverage surface were also decreased, but were of no significance.

Measure of Platelet Thrombus Volume

The dimensions of the thrombi formed in perfusions carried out at high shear rate with normal, FN-depleted plasma, and FN-depleted plasma to which FN had been added, were performed using the computerized system for morphometric evaluation. The results are shown in Fig 2. In perfusions performed with the annular chamber at a shear rate of 1,300 s^{-1}, the mean thrombus volume per surface area was 0.58 ± 0.11 $\mu m^3/\mu m^2$ for normal perfusates, and 0.12 ± 0.08 $\mu m^3/\mu m^2$ for FN-depleted perfusates, respectively.

Table 3. Platelet Deposition on α-Chymotrypsin–Treated Rabbit Vessels Preincubated With Purified FN

Vessel Wall Preincubation	Shear Rate (s^{-1})	Vessel Surface Covered (%)				
		Contact	Spread	Ag < 5μm	Thrombus	Surface Coverage
Buffer (n = 4)	300	2.9 ± 0.6	4.3 ± 0.9	2.0 ± 0.4	1.8 ± 0.5	11.0 ± 1.9
FN (n = 4)	300	3.1 ± 0.7	3.8 ± 1.0	2.3 ± 0.5	1.6 ± 0.4	10.8 ± 2.4
Buffer (n = 4)	1,300	3.9 ± 0.4	10.6 ± 2.5	3.0 ± 0.7	1.6 ± 0.1	17.2 ± 4.2
FN (n = 3)	1,300	4.1 ± 1.1	8.8 ± 2.2	2.1 ± 0.5	0.8 ± 0.4	15.0 ± 3.5

α-Chymotrypsin–digested rabbit vessels were preincubated with purified FN isolated from plasma by affinity chromatography and exposed to blood for ten minutes. Perfusates were prepared with FN-depleted plasma. Values represent mean ± SEM with the number of experiments in parentheses.

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Table 4. Platelet Deposition on Fibrillar Collagen Type III

Perfusate	Shear Rate (s^{-1})	Surface Covered (%)				
		Contact	Spread	Ag < 5μm	Thrombus	Surface Coverage
Normal plasma (n = 4)	300	0.6 ± 0.2	10.1 ± 2.1	14.2 ± 1.7	6.1 ± 1.7	31.0 ± 5.0
FN-depleted plasma (n = 3)	300	0.4 ± 0.2	4.2 ± 2.5	8.3 ± 0.8*	2.0 ± 0.7*	14.8 ± 3.7*
Normal plasma (n = 3)	1,300	0.2 ± 0.05	8.1 ± 2.1	36.9 ± 5.3	14.3 ± 3.0	59.5 ± 10.4
FN-depleted plasma (n = 3)	1,300	0.6 ± 0.01	9.0 ± 1.8	29.8 ± 3.5	3.6 ± 1.0*	43.0 ± 6.7

Fibrillar collagen type III was exposed to blood for ten minutes. Values represent mean ± SEM with the number of experiments in parentheses.
*$P < .01$.

These values reached the significant level ($P < .01$). Addition of purified FN to FN-depleted perfusates restored the value of thrombus volume to 0.48 ± 0.12 $\mu m^3/\mu m^2$.

Measure of thrombus volume in perfusions carried out with the flat chamber, using human fibrillar collagen as the substrate for platelet adhesion, displayed similar results. In this set of experiments, a statistically significant decrease ($P < .01$) in the mean thrombus volume per surface area was 0.67 ± 0.09 $\mu m^3/\mu m^2$ in normal perfusions and 0.20 ± 0.10 $\mu m^3/\mu m^2$ in FN-depleted perfusions was observed. The results obtained following the addition of 300 μg/mL of purified FN to FN-depleted perfusates showed a value of 0.60 ± 0.15 $\mu m^3/\mu m^2$, which was similar to the value

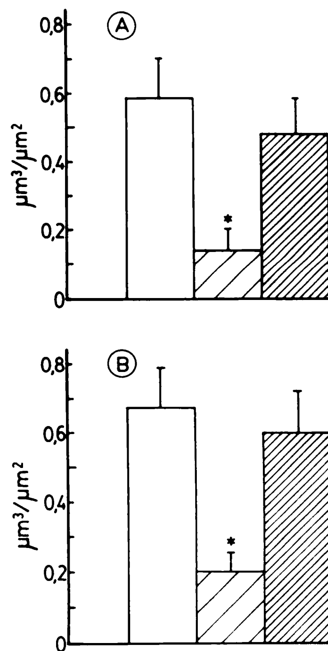

Fig 2. Effects of plasma FN on platelet thrombus formation Platelet thrombus volume per surface area was measured in ten minute perfusions at 1,300 s^{-1} and expressed in $\mu m^3/\mu m^2$. (A) Perfusions were performed with the annular chamber using α-chymotrypsin–digested rabbit segments. The number of experiments were n = 7, n = 6, and n = 3 for normal plasma, FN-depleted plasma, and FN-depleted plasma + FN, respectively. (B) Perfusions were performed with the flat chamber using fibrillar human collagen type III. The number of experiments were n = 4, n = 3, and n = 4 for normal plasma, FN-depleted plasma, and FN-depleted plasma + FN, respectively. □, Normal plasma; ▨, FN-depleted plasma; ▩, FN-depleted plasma + FN. Bars indicate mean thrombus volume per surface area and lines indicate SEM. $P < .01$.

measured in control perfusions. These results, using both perfusion systems, indicate the importance of plasma FN in platelet thrombus formation.

DISCUSSION

It is well documented that FN plays a role in some adhesion-mediated cell functions, including the interaction of platelets with vessel subendothelium.[15,23] In the present study, we investigated the role of plasma FN in platelet adhesion and platelet thrombus formation under flow conditions. To expose flowing blood to collagenous subendothelial and purified human collagen type III surfaces two perfusion systems were used. Platelet-subendothelium interaction was studied using annular chambers, with α-chymotrypsin–digested rabbit arteries, and flat chambers, with coverslips coated with fibrillar human collagen type III. Our results indicated that plasma FN is required for platelet aggregate and platelet thrombus formation, following adhesion in both types of surfaces studied.

The experiments carried out with α-chymotrypsin–treated rabbit vessels, particularly those performed at high shear rate, in which the percentage of thrombus formed is higher, indicate that plasma FN plays an important role in thrombus formation. The fact that average thrombus volume was significantly reduced in FN-depleted perfusions and that the addition of purified FN to the perfusates restored the values to those measured in control perfusions, strongly supports that plasma FN is not only important for the adhesion of platelets to subendothelium, but also for subsequent platelet thrombus growth under flow conditions.

Our results do not agree with those of Houdijk and Sixma,[23] who reported that FN in human artery subendothelium, but not in plasma, was important for platelet adhesion. However, more recently the same investigators found that platelet adhesion to endothelial cell matrix presented a shear-rate dependent requirement for plasma FN.[4] These differences in the relative role of plasma FN on platelet adhesion can probably be explained by differences between the surfaces used for platelet adhesion studies. In our studies, we used α-chymotrypsin–digested rabbit vessels that, as assessed by immunofluorescence staining (unpublished observation), were found to lack intact subendothelial FN, following enzymatic treatment. Preincubation of digested vessels with purified human FN did not enhance platelet deposition on subendothelium at any shear rate studied. This lack of effect may be due to human FN not incorporating into the collagen network of the digested rabbit vessels. This

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may be caused by differences between animal species or by steric hindrance due to remaining FN fragments.

The experiments performed with the flat chamber using fibrillar collagen type III as a substrate for platelet adhesion displayed similar results to those obtained with enzymatically digested vessel subendothelium. In this system, plasma FN was also found to be required for platelet adhesion and platelet thrombus formation on collagen coated surfaces, at both low and high shear rates. The observation that in both experimental systems used, addition of purified FN to the perfusates restored the values obtained for control perfusions indicating that the effects on platelet deposition were due to depletion of plasma FN.

Until now other plasma proteins such as fibrinogen[24] and von Willebrand factor[25] have been suggested to play a role in platelet-platelet interaction. There is evidence that fibrinogen, von Willebrand factor, and FN bind to the GPIIb/IIIa membrane complex of activated platelets. Recently, various investigators explored the role of GPIIb/IIIa in platelet adhesion to subendothelium, using the Baumgartner perfusion system. Sakariassen et al[12] showed a defect in platelet adhesion at high shear rates after blocking the GPIIb/IIIa complex with specific monoclonal antibodies. Furthermore, Weiss et al[13] reported that platelets deficient in GPIIb/IIIa do not spread normally on the subendothelial surface. However, the specific binding site of FN to the GPIIb/IIIa receptor and its implications in platelet function are still not well understood. Thus, it has been reported that FN binds to specific divalent cation-dependent receptors on washed, thrombin stimulated platelets,[26] but does not bind to ADP- or epinephrine-stimulated platelets, whereas, fibrinogen and von Willebrand factor do.[27] These observations suggest separate binding sites for FN in the platelet membrane. Moreover, Dixit et al[28] described a monoclonal antibody directed against FN that does not interfere with the function of the cell binding domain of the FN. The ability of this antibody to inhibit platelet aggregation suggests that there is a functional site present in FN that has a direct role on platelet aggregation. Furthermore, the results reported by Parise and Phillips[29] demonstrate that FN can bind directly to purified GPIIb/IIIa complex, strongly suggesting that it has the potential to function as a platelet receptor for FN.

Our results indicated that under flow conditions plasma FN, in addition to supporting platelet adhesion to collagenous subendothelial surfaces and to purified collagen type III, influences platelet thrombus formation. It is feasible that binding of FN to GPIIb/IIIa, or to another putative receptor present in activated platelets, favors interaction between platelets and contributes to the development of platelet thrombi. In conclusion, the results we present here indicate that plasma FN is one of the adhesive proteins required for platelet thrombus growth under flow conditions.

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