Decreased Plasma Fibronectin Leads to Delayed Thrombus Growth in Injured Arterioles

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Objective—Plasma fibronectin (FN) is decreased in several clinical conditions. We were interested to study the thrombotic and hemostatic consequences of the decrease in plasma FN (pFN), the role of FN splice variants in thrombosis, and to examine whether pFN incorporates into thrombi in vivo.

Methods and Results—We compared the thrombotic response to a vessel injury in FN heterozygous (FN+/−) mice and corresponding FN+/+ mice. Although normal thrombosis in venules was observed, a decrease to half in the pFN concentration in FN+/− mice caused a delay in the appearance of thrombi in arterioles and consequently a delay in their occlusion. We were able to rescue the thrombotic defect in the FN+/− mice by infusion of rat pFN. Additionally, we could show intense incorporation of fluorescent pFN-coated microspheres into the developing thrombi. Moreover, we found that mice expressing FN without the EIIIA or EIIIB domains specific to cellular FN including platelet FN had no thrombotic defect.

Conclusions—Mice heterozygous for FN have a striking defect in thrombus initiation and growth in arterioles attributable to the decrease of pFN. Our study is an example of haploid insufficiency for FN, and it emphasizes the fundamental role of this plasma protein in thrombosis in the arterial system. (Arterioscler Thromb Vasc Biol. 2006;26:1391-1396.)

Key Words: plasma fibronectin • arterial and venous injury • thrombus • fibronectin splice variants • intravital microscopy

Platelet adhesion to the subendothelium of an injured vessel wall and subsequent aggregation is a complex and critical event in platelet plug formation that requires the contribution of several adhesive proteins. One of these proteins is fibronectin (FN), which, together with von Willebrand factor (VWF) and fibrinogen, belongs to a group of major ligands thought to mediate platelet adhesion and aggregation.

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FN is a dimeric multidomain glycoprotein that exists in plasma and in tissue extracellular matrix.1 The largest pool of soluble FN in the adult mammal is in plasma (plasma FN [pFN]). pFN is produced specifically by hepatocytes and is present at 300 µg/mL in human plasma and 580 µg/mL in mice.2 Cellular FN is found in an insoluble multimeric form in the extracellular matrix of many tissues. In platelets, both forms of FN are present: the soluble form taken up by platelets from plasma and the cellular form coming originally from megakaryocytes. However, platelets have no cell surface FN in their resting state.3

FN performs a remarkably wide variety of activities: it plays a role in cell adhesion, migration, growth, and differentiation. FN is critically important in development, as demonstrated by early embryonic lethality in mice with targeted inactivation of the FN gene.2 It is a ligand for many members of the integrin receptor family, and it can bind to a number of biologically important molecules, including fibrin, heparin, and collagen.1 FN RNA is alternatively spliced at 3 regions: EIiIA(EDA), EIiIB(EDB), and V(CS-1). The alternatively spliced variants of FN containing the EIiIA and EIiIB splice variants are prominently expressed around developing blood vessels during embryonic growth.4 In adults, EIiIA and EIiIB splice variants are downregulated but still mainly expressed in the walls of blood vessels.5 EIiIA-null and EIiIB-null mice are viable and fertile, suggesting that embryonic vessel formation occurs normally in the absence of these splice variants.6–8 In normal plasma, most of the pFN lacks the EIiIA and EIiIB domains. However, both are present in plasma of patients with vascular injury or trauma9 and in mice with chronic inflammation (apolipoprotein E–deficient mice).8

Recently, our group reported that pFN is one of the ligands supporting platelet thrombus formation in vivo. In a model of arterial thrombosis, mice with complete deficiency of pFN...
(pFN conditional knockout mice) had delayed thrombus formation and growth and subsequent occlusion of injured arterioles. In this study, we asked whether a decrease in FN expression in the absence of interferon (IFN) would affect thrombus formation under arterial or venous flow conditions. This is important because reduced, but not absent, pFN is common in patients with liver disease and after trauma or surgery. We also wished to visualize pFN incorporation into the growing thrombi in vivo. Additionally, we were interested whether absence of EIIIA or EIIIB splice variants of cellular FN would affect thrombosis at high shear rate after acute vessel injury.

Methods

Mice
FN+/−, EIIIA+/−, EIIIB+/− (C57BL/6J background), and FN conditional knockout mouse with corresponding control mouse (FNflox/FNflox;MxCre+ and MxCre−) and their treatment with polyI-polyC were described previously. The heterozygous and corresponding wild-type (WT) mouse were produced by heterozygous FN+/− and FN+/+ crosses. Age-matched control WT mice corresponding to EIIIA+/− and EIIIB+/− on C57BL/6J background were purchased from Jackson Laboratory (Bar Harbor, Me). The experimental procedures were performed at the CBR Institute for Biomedical Research and approved by its animal care and use committee.

Determination of Whole Blood Clotting Time
Whole blood clotting time was measured as described with some modifications. Blood was collected into tubes containing 3.8% sodium citrate by cardiac puncture of the anesthetized mice. A total of 280 μL of whole blood was added to 20 μL of CaCl2 in 37°C precooled cuvettes. The onset and clotting time were measured by Sonoclot Coagulation and Platelet Function Analyzer (Sienco).

Bleeding Time Measurement
FN+/+ and FN+/− mice (9 to 10 weeks old) were anesthetized with ketamine/xylazine (150/15 mg/kg, IP), and a 3-mm segment of the tail was amputated. The tail was immersed in 0.9% isotonic saline at 37°C, and the time required for the stream of blood to stop was defined as the bleeding time.

Detection of FN
Platelets were isolated from pooled blood of 2 to 3 mice, and the same number of platelets was lysed for each genotype in 3 independent experiments. The plasma samples were obtained from single mice (3 per each group), and the same volumes were tested. Platelet and plasma samples were then analyzed by Western blot using a rabbit antibody against human FN with 1:3000 dilution (Sigma) followed by chemiluminescent detection (Amersham Biosciences) and quantified by using a PhosphorImager. pFN levels in FN+/+, FN+/−, EIIIA+/−, and EIIIB+/− mice were additionally quantified by radial immunodiffusion using an antibody to human FN. The levels of pFN in mice of C57BL/6J background were entirely comparable to those in mice on mixed background C57BL/6J129sV.2

In Vivo Thrombosis Model
The arterial thrombosis model was described previously. Briefly, platelets from donor mice (>5 weeks old) were isolated from platelet-rich plasma and fluorescently labeled with calcein acetoxymethyl ester (Molecular Probes). The recipient mice (3 to 4 weeks old; weighing 12 to 15 g) were orbitally injected with the fluorescently labeled platelets of matching genotype. A subgroup of FN+/− mice was then injected with rat pFN (Sigma; 290 μg per mL of blood). Under anesthesia induced with intraperitoneal injection of tribromoethanol (Fluka; 0.15 mg/10 g of body weight), the mouse mesentery was then exteriorized through a midline abdominal incision. FeCl3 (Sigma; 10 μL of 250 mmol/L solution) was applied topically to an arteriole that induced local vessel injury and denudation of the endothelium. For arterial thrombosis in EIIIA+/−, EIIIB+/−, and corresponding WT mice as well as for venous thrombosis, we used filter paper soaked in 10% FeCl3, which was placed for 5 minutes topically on the mesenteric vessels to induce the injury. Vessels were monitored for 40 minutes after injury or until full occlusion occurred (blood flow stopped for >30 s). If the vessel did not occlude, 40 minutes was taken as the occlusion time for data analysis.

In Vivo Detection of pFN in the Thrombus
Yellow green (excitation/emission: 505 nm/515 nm) and red (excitation/emission: 580 nm/605 nm) carboxylate-modified microspheres (1.0 μm diameter; Molecular Probes) were covalently coupled to rat pFN (Sigma) and control rat IgG1 (BD Pharmingen), respectively. A total of 250 μg protein in 0.5 mL 50 mmol/L 2-[N-Morpholino]ethane sulfonic acid buffer, pH 6.0, was coupled to 6.75 × 106 microspheres according to manufacturer instructions (Molecular Probes). In the first experimental setting, WT C57BL/6 mice were infused with 2 sets of microspheres, pFN and control IgG, coated with different colors (0.5 × 106 microspheres per mouse) 5 minutes after the vessel injury was induced by FeCl3. Mesenteric arterioles and venules were observed immediately thereafter by fluorescence intravital microscopy. In another experimental setting, the incorporation of pFN-coated microspheres (yellow green) specifically into thrombi was verified by using the calcein-labeled platelets (red—orange; Molecular Probes) injected into the same recipient mouse.

Statistical Analysis
Data are presented as mean±SEM. Statistical significance was assessed by unpaired Student t test (for comparison of 2 groups) or by ANOVA with the Bonferroni test if >2 groups were compared.
Western blot (Figure 1) in 21% reduction in platelet FN content as determined by Western blot and confirmed by radial diffusion assay) and a

Results

Plasma and Platelet FN, Whole Blood Clotting, and Bleeding Time in FN+/+ Mice

pFN is an important component of fibrin clots.1 To evaluate whether a 55% reduction in pFN levels (determined by Western blot and confirmed by radial diffusion assay) and a 21% reduction in platelet FN content as determined by Western blot (Figure 1) in FN+/− mice affect hemostatic parameters, we measured the whole blood clotting time, clot rate, and bleeding time. The onset and the clotting rate of whole blood in vitro were not significantly different in FN+/− mice compared with control mice. Additionally, both groups of mice were able to control their blood loss without cauterization after amputation of a 3-mm portion of the tail. Thus, the bleeding time and clot formation were not significantly impaired in FN+/− mice (Table).

Early Platelet Adhesion and Thrombus Formation in Arterioles of FN+/− Mice

To evaluate whether a decreased level of pFN affects platelet adhesion and thrombus formation in arterioles, we superfused ferric chloride on the vessel and observed the behavior of fluorescent platelets by intravital microscopy. The numbers of single platelets, adhering during the interval 2 to 3 minutes after the vessel injury, were not significantly different in FN+/− compared with FN+/+ mice (38±7 in FN+/− versus 52±9 in FN+/+; P>0.05). On the other hand, we found a striking delay of several minutes in appearance of the first thrombus in the injured arterioles in FN+/− mice when compared with FN+/+ (14.2±1.04 minutes in FN+/− versus 5.7±0.12 minutes in FN+/+; P<0.001). We observed that the thrombi grew very slowly in the FN+/−, although they were stably anchored to the vessel wall. The growth of the thrombus was slowed down by constant platelet shedding (Figure 2).

Occlusion of Arterioles and Thrombus Embolization in FN+/− Mice

The lack of firm adhesion of the platelets to the thrombus led to occlusion delay in FN+/− mice (36.8±2.7 minutes in FN+/− versus 28±1.6 minutes in FN+/+; P<0.01). The difference in occlusion time is an underestimate because the majority of arterioles in the FN+/− mice were not occluded by the 40 minutes observation period. In contrast, all FN+/+ mice occluded before this time point (Figure 3A). Despite the constant shedding of single platelets or small platelet clumps in FN+/− mice, the thrombi did not produce many large emboli of diameter >30 μm. Thus, the frequency of large emboli in FN+/− mice was not different from FN+/+ mice (2.25±0.62 in FN+/− versus 2.6±0.6 in FN+/+; P>0.05).

Infusion of Rat pFN in FN+/− Mice Resulted in Normal Thrombus Growth

to determine whether it was the decrease in plasma or cellular FN that was responsible for the thrombotic defect in FN+/− mice, we reconstituted the pFN to WT levels by infusion of rat pFN. The level of pFN in FN+/− mice was determined before infusion and after infusion/intravital microscopy in the same mice by Western blot (Figure 1A). The normalization of pFN level in FN+/− mice prevented platelet shedding from thrombi and produced occlusion of injured arterioles in all animals (Figure 2, bottom panels; Figure 3A).

Thrombogenesis in Venules of FN+/− Mice

Because the injured arterioles of FN+/− mice had such a striking thrombosis phenotype, we were interested to assess the venous thrombogenesis as well and have developed a venous thrombosis model for this purpose. We investigated the thrombus growth by observing FeCl3-injured venules of 200 to 300 μm in diameter. The growth of thrombi at low venular shear rate was not affected in FN+/− mice with venules occluding at similar time points compared with control mice (22±4 minutes in FN+/− versus 20±2 minutes in FN+/+; P>0.05; Figure 3B). Because this was not shown previously, we also studied the venous thrombosis in FN
conditional knockout mice. We found that complete deficiency of pFN did not affect the occlusion of venules either (28±4 minutes in pFN conditional knockout mice [n=7] versus 25±3 minutes in corresponding control mice [n=9]; $P>0.05$).

In Vivo Incorporation of FN Into Thrombus
To visualize incorporation of FN into the growing thrombi in the injured vessels, we injected WT mice with pFN coupled to fluorescent microspheres (1 μm diameter) together with microspheres of a different color coupled to a control protein (IgG). The injected pFN microspheres were seen binding to thrombi growing in both mesenteric arterioles and venules as well as to injured vessel wall with denuded endothelium (Figure 4A). Control protein (IgG) coupled to microspheres did not bind to the thrombi nor to injured vessel wall (Figure 4B). In another study, we showed that fluorescently labeled platelets codistributed with the pFN microspheres incorporated into thrombi (Figure 4C and 4D).

Thrombosis in Arterioles of Mice Lacking the Alternatively Spliced EIIIA or EIIIB Domains of FN
We investigated the effect of absence of cellular FN splice domains on thrombosis using $EIIIA^{−/−}$ and $EIIIB^{−/−}$ mice. The absence of either splice domain of FN did not affect plasma levels of FN, as determined by Western blot (Figure 5A) and by radial immunodiffusion (data not shown). The growth of thrombi in arterioles covered for 5 minutes with FeCl$_3$-soaked filter paper was not affected by EIIIA or EIIIB deficiency, with occlusion times similar to WT (13.2±1.3 minutes in $EIIIA^{−/−}$; 12.1±1.3 minutes in $EIIIB^{−/−}$ versus 12±0.8 minutes in WT; both $P>0.05$; Figure 5B). Thus, the alternatively spliced EIIIA and EIIIB domains, present in cellular and platelet FN, do not play an important role in thrombus formation.

Discussion
The studies presented here underscore the importance of pFN in the process of arterial thrombus formation. In the FeCl$_3$-induced thrombosis model, we observed significantly delayed thrombus formation in arterioles after vessel wall injury despite normal platelet tethering in $FN^{+/−}$ mice compared with $FN^{+/+}$ mice. Furthermore, we noted constant shedding of platelets from the growing thrombi, which eventually led to occlusion delay, with the majority of arterioles in $FN^{+/−}$ mice remaining patent. The occlusion defect was successfully rescued in $FN^{+/−}$ by infusion of rat pFN in $FN^{+/−}$ that normalized the pFN levels.

The defect in thrombosis in the $FN^{+/−}$ mice strikingly resembles the phenotype observed in vivo by intravital microscopy in mice with an induced complete deficiency of pFN (pFN conditional knockout mice). However, the $FN^{+/−}$ mouse offers several advantages over the pFN conditional knockout model for studies of the role of FN in thrombosis. First, the $FN^{+/−}$ mouse model reflects a more physiological decrease of pFN. Indeed, the reduced plasma level, but not complete deficiency of FN, is common in several disease states. Patients with liver disease, renal failure, sepsis, and after trauma or surgery have been found to exhibit low concentrations of pFN, which may contribute to the hemostatic defects so often seen in these patients. Second, we did not have to induce IFN production by treatment with polyI-polyC (IFN inducer used in the pFN conditional knockout mouse) to excise the FN gene. This is important because both polyI-polyC and IFN could affect platelet function. This all makes the FN heterozygous mouse model attractive for studying the role of FN function in vivo.

Using a FeCl$_3$-induced vessel wall injury model, we examined the pFN incorporation into the growing thrombi in
WT mice. Fluorescently labeled pFN microspheres infused into a mouse specifically localized within the developing thrombi in arterioles and venules, indicating that pFN becomes an integral part of the thrombus at both high and low shear rate conditions.

Several known interactions of pFN, for example with fibrin, collagen, and activated platelets, could be of importance in thrombus formation after injury. During coagulation, soluble FN is cross-linked to fibrin molecules by Factor XIIIa transglutaminase, which enhances the stability of the clot.19 Furthermore, incorporation of FN into fibrin augments the growth of thrombi in vitro.20 We observed no difference in clotting in FN+/− mice compared with WT mice. Our results indicate that the polymerization and gelation of fibrinogen is not affected by the partial reduction in FN levels. This is in line with the results of in vitro plasma clotting activity measurement in pFN conditional knockout mice.10 Furthermore, the case of a family with low pFN levels has been reported with no abnormal hematologic laboratory tests.21

Activated platelets have the ability to bind exogenous pFN through integrins αIIbβ3 or α5β1 expressed on their surface. This happens in Arg-Gly-Asp (RGD) sequence-dependent and -independent manners.22,23 The platelet membrane receptor αIIbβ3, which is a key integrin in thrombus formation, is known to bind fibrinogen and VWF in addition to pFN and to promote the internalization of fibrinogen24 and FN into α-granules of platelets.13 Recently, it was demonstrated that FN can engage αIIbβ3 in a similar manner to fibrinogen. The engagement is associated with an activation of signaling events, including tyrosine phosphorylation of focal adhesion kinase and phospholipase Cy2 with intracellular mobilization of calcium.25 This outside-in signaling induced by FN through αIIbβ3 might be ultimately necessary for full platelet spreading, aggregation, and effective hemostatic plug formation. In vivo, this optimal outside-in signaling by FN might help to glue the platelets to each other during thrombus formation at high shear rates, a process most defective in mice with reduced pFN.

In vitro experiments suggested that pFN stimulates the binding of platelets to collagen26 and to subendothelium both under arterial and venous flow conditions.27 Additionally, the cellular FN normally present in subendothelium can, during vessel injury, contribute to platelet adhesion as shown in vitro.28 In the FeCl3-induced injury model, we did not observe a significant decrease of early platelet interaction with subendothelium in FN+/− compared with FN+/+ mice, indicating that significant reduction in pFN and also cellular FN do not affect this process. In the arterial system, in which the interaction of vWF with GPIb is of critical importance, pFN or cellular FN likely plays only a secondary role in the initial platelet adhesion.

In our model, thrombogenesis at low shear rate was not influenced by a decrease of FN in plasma. The role of FN in thrombosis appears shear dependent, with a prominent role at arterial shear rate. This is very similar to the thrombosis phenotype seen in vessels of CD40L−/− mice, which present thrombus instability specifically at arterial shear rate.29 Interestingly, the CD40L−/− mice, like FN+/− mice, exhibit normal bleeding time.29 CD40L is known to stabilize arterial thrombi by a β3 integrin-dependent signaling,29 and this could also be the case for FN.

Although the decrease of pFN in FN+/− mice (as described in this study) and pFN deficiency40 are associated with impaired arterial thrombosis, elevation of pFN may have the opposite effect.20 High levels of pFN have been found in both patients and experimental animals with thrombotic acute coronary syndromes.30,31 Thus, information about pFN levels might provide insights as to the probability of arterial thrombotic events. Our results suggest that the thrombosis defect in FN+/− mice was attributable to a decrease in pFN and not cellular FN. This conclusion is based on observations: first, the restoration of pFN up to WT level corrected the thrombosis in arterioles. The absence of either EIIIA or EIIIB isoforms of FN does not affect the plasma level of FN or thrombosis in arterioles. The absence of either splice domain of FN did not affect pFN level, as evaluated by Western blot and expressed as percentage of WT (n=3; P>0.05 for both EIIIA−/− and EIIIB−/− vs WT; A). All injured arterioles in WT, EIIIA−/−, and EIIIB−/− mice occluded at similar time points, and no statistical differences were observed (B).

**Figure 5.** The absence of EIIIA or EIIIB domains in FN does not affect the plasma level of FN or thrombosis in arterioles. The absence of either splice domain of FN did not affect pFN level, as evaluated by Western blot and expressed as percentage of WT (n=3; P>0.05 for both EIIIA−/− and EIIIB−/− vs WT; A). All injured arterioles in WT, EIIIA−/−, and EIIIB−/− mice occluded at similar time points, and no statistical differences were observed (B).

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