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Emerging roles of fibronectin in thrombosis

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Abstract

Fibronectin (FN) is a glycoprotein recognized originally in the 1940s as a contaminant of fibrinogen in Cohn fraction I of plasma. Decades of research demonstrated FN synthesis by a variety of cells and defined FN as an essential component of the extracellular matrix with roles in embryogenesis, development, and wound healing. More recently, FN has emerged as player in platelet thrombus formation and diseases associated with thrombosis including vascular remodeling, atherosclerosis, and cardiac repair following a myocardial infarct. We discuss the mechanisms by which this might occur and conclude that FN may have a unique role in thrombosis without affecting normal hemostasis and therefore may be a reasonable therapeutic target for the prevention of thrombotic diseases.

Introduction

Fibronectin (FN) is an approximately 500-kDa glycoprotein dimer made of two nearly identical ~250 kDa subunits that are connected by disulfide bonds near the C-termini. Each monomer is composed of repeating type I (12), type II (2), and type III (15-17) modules(1). Type III modules are used in many different proteins across a wide range of species. In FN, type III modules interact with substances known to be involved in hemostasis, perhaps most significantly with integrins via sequences including the RGD motif in the tenth type III module (¹⁰F3)(1). Type II modules are less common, and type I modules are only found in vertebrates where, in addition to the 12 examples in FN, single modules are found in tissue plasminogen activator, factor XII, and hepatocyte growth factor activator. The evolutionary origin of the type I module is likely the much more widespread von Willebrand factor-C module(2). Type I modules have a major β -sheet, a minor β -sheet, and two conserved disulfide bonds, one of which is a staple-type, potentially allosteric disulfide that connects adjacent β -strands(3). Type I modules interact with many substances known to be involved in hemostasis including fibrinogen and fibrin and heparin/heparan sulfate(1).

Human FN is encoded by a single 75-kb gene that is located on chromosome 2q34. Through alternative mRNA splicing, this gene encodes 20 different FN variant subunits. Variability comes from inclusion or exclusion of two type III modules, EIIIA and EIIB (also called EDA and EDB), as well as within the type III homology connecting segment in which splicing by exon subdivision leads to five different versions of a 120-residue sequence (the so-called variable region)(4).

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Detergent-insoluble FN fibrils, which are formed in a process known as FN assembly, are a major component of provisional or renewing extracellular matrix of tissues. Some matrix FN is synthesized locally by cells, including fibroblasts, and is rich in EIIIA or EIIB. The remainder of FN in tissues originates in the plasma(5). FN binds to sites at the periphery of adherent cells via the type I modules and then engages integrins via the type III modules(6). The fibrillar adhesion proteins tensin and $\alpha 5\beta 1$ integrin translocate from focal adhesions in a process that is thought to extend bound FN and allow FN-FN interactions to occur(7).

Plasma and platelet FN

In humans, soluble plasma FN circulates at 230 to 650 $\mu\text{g mL}^{-1}$ (8). Plasma FN is secreted by hepatocytes(9). It is synthesized at 10-20% the rate of fibrinogen and has a half-life of two days(10). In healthy people, plasma FN lacks EIIIA and EIIB and is generally a heterodimer in which one subunit must contain part or all of the variable region. This is because mRNA in hepatocytes lacks the exons for EIIIA and EIIB, and there is selective secretion of the dimers in which one subunit lacks any residues from the variable region and the other has a variable region insert(4,11). In mice in which the liver is forced to express only EIIIA-containing FN, plasma FN levels are decreased(5,12). Atherosclerosis, acute vascular injury, and ischemic stroke are associated with several percent of plasma FN containing EIIIA(13-15). The source of plasma EIIIA containing-FN in these pathologic conditions (e.g., more local synthesis or change in hepatocyte mRNA splicing) is not known.

Platelets contain FN in α -granules at a concentration of approximately 1 μg per 3×10^8 platelets, *i.e.*, platelets in 1 mL of blood, along with thrombospondin, fibrinogen, von Willebrand factor, and vitronectin(16). Upon activation, this FN is released. Although the contribution of platelets to overall plasma FN content is small, platelet FN may have special effects because it contains EIIIA and variable regions similar to cellular FN(17). The presence of EIIIA also suggests that some platelet FN is synthesized and packaged in megakaryocytes. Some packaged FN, however, is likely taken up by endocytosis the way that fibrinogen is; lack of competition of the endocytic pathway presumably is why the content of platelet FN is greater in mice unable to internalize fibrinogen(18).

FN does not affect normal hemostasis

Data from both humans and mice indicate that FN does not alter normal hemostasis. One family has been identified with a mild deficiency in plasma FN levels ($100 \mu\text{g mL}^{-1}$). Affected members had normal hemostatic parameters(19). Further, mice homozygous for deficiency in plasma FN have normal tail bleeding times and activated partial thromboplastin times(20).

Despite the importance of the conversion of fibrinogen to fibrin by thrombin and fibrinogen binding to platelets for normal hemostasis, patients deficient in fibrinogen may have only a mild bleeding phenotype(21), which suggests that fibrinogen's actions are compensated for by other proteins. Further, FXIII deficiency causes a severe bleeding phenotype including spontaneous intracranial hemorrhages(22), suggesting that substrates in addition to fibrin, *i.e.*, FN and $\alpha 2$ -antiplasmin, are important for normal hemostasis. Current data suggests that FN acts in concert with fibrinogen/fibrin rather than substitutes for fibrinogen/fibrin. FN is organized into fibrils by adherent platelets(23), but these fibrils are very different from the space-filling fibrin meshwork to which FN is covalently cross-linked by thrombin-activated factor XIII(24). Analysis of platelets from a hypofibrinogenemic patient showed increased levels of platelet FN, but only trace amounts of FN were seen on the surface of the patient's platelets after activation with thrombin(25). Further, when a thrombin receptor activation peptide that prevents conversion of fibrinogen to fibrin was used to stimulate normal platelets, very little FN was seen on the surface of aggregated platelets(25). This suggests that association

of FN with the surface of aggregating platelets is to fibrin rather than to $\alpha\text{IIb}\beta_3$, as with fibrinogen.

Further evidence that FN does not compensate for fibrinogen comes from studies comparing the triple knock-out of plasma FN, fibrinogen, and von Willebrand factor with the von Willebrand factor/fibrinogen double knock-out. This animal was engineered to test the hypothesis that FN supported the robust platelet plug formation found in the double knock-out mouse lacking fibrinogen and von Willebrand factor(26). Surprisingly, intravital microscopy showed that triple knock-out mice had increased rather than decreased thrombus growth compared to the double knock-outs(27). Thus, in the absence of von Willebrand factor and fibrinogen, FN seems to regulate thrombus formation negatively.

Association of human and mouse plasma FN level with thrombosis

So far, little insight into pathophysiologic roles of FN has emerged from studies of genetic changes in humans. Mutations in type III modules of FN have been implicated in familial glomerulonephritis associated with excess FN deposition in the kidney(28). The deposits may represent aggregation of unstable FN rather than excess deposition of FN into the extracellular matrix. There is no information about thrombosis. No reference was made to thrombotic events for the family with a mild deficiency in FN(19).

A positive correlation between FN levels and the ability to promote thrombus formation was found in an *in vitro* flow chamber model of thrombosis, where FN perfused with human platelets and red blood cells at concentrations ranging from 10 to 600 $\mu\text{g mL}^{-1}$ caused a dose-dependent increase in thrombus volume and the number of platelets adherent to a coating of FN-fibrin(29). Inclusion of the increasing concentrations of FN during formation of the FN-fibrin coating caused a further increase in platelet thrombus volume(29) (Fig. 1). By changing both FN concentrations during formation of the coating and in the perfusate, 10-20-fold differences in platelet thrombus deposition are evident after 5 min of flow. Fluorescent imaging demonstrated incorporation of FITC-FN into thrombi. Effects of FN on thrombus build-up were blocked by FUD(29), a 49-residue sequence derived from the F1 adhesin of *Streptococcus pyogenes* that interacts with N-terminal region of FN and inhibits FN assembly(30).

Several studies have looked at the relationship between the level of plasma FN and coronary artery disease. Comparison of studies is complicated by differential timing of FN measurements in relationship to disease and different exclusion criteria for patients on pharmacologic agents. At least four groups found increased levels of FN associated with coronary artery disease(31-34), one group found lower levels of FN associated with coronary artery disease(35), and one study found no difference between FN levels in patients with coronary artery disease and the control group(36). In one study, males with venous thromboembolism had significantly higher FN levels than controls(37). Values from studies are located along the flat part of the curve of thrombus volume versus FN concentration in the purified system (Fig. 1).

Studies in mice, which have the same wide range of FN concentrations as humans(38), indicate that platelet thrombus formation is not a simple function of plasma FN concentration. The level of mouse plasma FN ranges in a strain-specific manner in 24 strains of mice from 7 genetic families from 121 to 414 $\mu\text{g mL}^{-1}$ (38). *In vivo* studies of C57BL/6J mice homozygous for conditional knock-out of plasma FN and, surprisingly, heterozygous for conditional knock-out of plasma FN indicate that low levels of plasma FN impair thrombus formation(39,40). The FN levels in normal and heterozygotes are 140 and 70 $\mu\text{g mL}^{-1}$ respectively(38). After ferric chloride injury of arterioles, platelets from the deficient mice initially adhered normally, but there was a delay in thrombus formation. Thrombi continuously shed platelets, which increased the time to vessel occlusion(40). The defect in thrombus formation was alleviated when rat FN

was perfused in these mice(39). Thus, low levels of plasma FN are associated with thrombus instability in mice. However, no correlation is evident between published plasma FN concentrations ranging from 140 to 352 $\mu\text{g mL}^{-1}$ and the time to vessel closure after injury when comparing studies of five strains of mice(38,41).

C57BL/6J mice constitutively expressing only FN with EIIIA had an increase in thrombus formation despite a 60-80% reduction in levels of plasma and platelet FN(12). The observation that there is an increase in thrombus formation despite low levels of FN in the plasma suggests that EIIIA-containing FN is particularly efficacious in initiating or incorporating into a growing thrombus. The mechanism behind the prothrombotic effect of EIIIA is not known, but it could be related to the known propensity of EIIIA FN to deposit into fibrils(42), the presence of recognition sites for $\alpha 9\beta 1$ or $\alpha 4\beta 1$ (integrins that are not on platelets, but are on leukocytes or endothelial cells) in EIIIA, or alteration of an important structural element of FN such as the RGD motif in the tenth type III module(4,42).

In considering the relationship between level of plasma FN and thrombosis, it is necessary to consider degradation of FN. FN is susceptible to proteolysis by numerous proteases. Degradation of FN is associated with inflammation and has been implicated in the pathogenesis of inflammatory lung diseases including emphysema and pulmonary fibrosis(43). Inflammation is increasingly recognized as a contributor to atherosclerosis and thrombosis (44). A study comparing French centenarians and sick, elderly adults found a lower level of FN degradation products in the plasma of healthy centenarians(45).

Role of FN in platelet thrombus formation

Thrombus formation involves platelet adhesion, platelet activation and aggregation, and build-up of a fibrin-rich thrombus(46). Consistent with the idea that FN works together with fibrinogen/fibrin, the first recognized means in which FN could contribute to thrombus growth and stability is by binding and covalent cross-linking to fibrin. FN is cross-linked to fibrin via the transglutaminase FXIII. FXIII is calcium dependent and forms ϵ -(γ glutamyl) lysine bonds between lysines within at least the $\text{A}\alpha$ -chain of fibrin and a glutamine in FN(24). The cross-linking process occurs independently of platelets. However, in a flow chamber, FN cross-linked to fibrin by FXIII provides a substrate for increased platelet adhesion and thrombus growth (29).

FN has the possibility of mediating platelet adhesion by three RGD-recognizing integrins on platelets- $\alpha\text{IIb}\beta 3$, $\alpha\text{v}\beta 3$, and $\alpha 5\beta 1$. *In vivo*, ligands mediating platelet tethering to injured vessels include von Willebrand factor binding to glycoprotein Ib-V-IX and type 1 collagen binding to glycoprotein VI(46). Whether FN contributes to platelet adhesion in physiologic or pathophysiologic situations, such as atherosclerotic plaque rupture, is not known. FN is less efficient at mediating platelet adhesion than von Willebrand factor or type 1 collagen under shear conditions(16). However, studies showing that antibodies or Fab fragments against FN decrease platelet adhesion to the subendothelium and that removing FN from the perfusate decreases platelet adhesion to the subendothelium or fibrillar type III collagen, suggest that FN contributes to platelet adhesion under shear conditions, as recently reviewed(16). *In vitro*, under static conditions, platelets adhere to absorbed FN and under flow conditions, lysophosphatidic acid-treated platelets adhere to absorbed fibrin, FN, and FN cross-linked to fibrin(29). Static adhesion is mediated by $\alpha\text{v}\beta 3$, $\alpha\text{IIb}\beta 3$, and a $\beta 1$ integrin (likely $\alpha 5\beta 1$), likely through the RGD motif in $^{10}\text{F3}$ (47). It is possible that regions of FN outside $^{10}\text{F3}$, including the NGR sequences in the N-terminal 70 K region that spontaneously isomerize to *iso*DGR, also contribute to platelet adhesion to FN(48).

Since FN is a dimer, it is reasonable to hypothesize that FN, like fibrinogen, could mediate platelet aggregation by binding integrins and tethering adjacent platelets or that FN could bind

to integrins and block fibrinogen-mediated platelet aggregation by competing for $\alpha\text{IIb}\beta 3$. Evidence exists for both scenarios. Addition of FN to washed platelets blocks platelet aggregation in response to thrombin, collagen, or the calcium ionophore A23187 (49,50) whereas monoclonal antibodies against FN block platelet aggregation in response to low dose collagen or thrombin and in one study A23187(51,52).

Fluorescent and electron microscopy studies showed that FN binds to the surface of adherent platelets and, as with fibroblasts, FN can be assembled by platelets into a matrix(23). Under static conditions, platelets adherent to FN, fibrin, laminin-111, and type 1 collagen assemble FN fibrils on their surface whereas platelets adherent to vitronectin, fibrinogen, and von Willebrand factor do not(29,47,53,54). The difference between fibrin and fibrinogen may be due to decreased accessibility of the C-terminal $\alpha\text{IIb}\beta 3$ -binding sequence upon polymerization and cross-linking of fibrin(47). As mentioned above, in an *in vitro* flow chamber model of thrombosis, FITC-FN binds to lysophosphatidic-acid treated platelets adherent to FN or FN cross-linked to fibrin and increases platelet adhesion and thrombus volume(29). At this point there is not a satisfactory explanation as to why adhesive ligands differently support platelet FN assembly. However, FN-null fibroblasts are subject to a similar substrate regulation of FN assembly(55).

Many details regarding the molecular mechanisms of FN assembly mediated by platelets remain unknown. Platelet agonists, including ADP, which is released upon platelet activation, increase the assembly of FN on the surface of platelets adherent to FN(23). Further, like fibroblast FN assembly, platelet FN assembly requires an intact actin cytoskeleton and diverse signaling molecules including phosphoinositol-3 kinase and protein kinase C(54).

A question relevant to FN assembly by both platelets and fibroblasts is the relative importance of FN's integrin binding RGD sequence in $^{10}\text{F3}$ and the N-terminal 70 K region (70 K FN) in the initial interaction between FN and the cell surface. Support for the 70 K FN in initial binding comes from studies showing that in solution the 70 K region, but not the RGD sequence in $^{10}\text{F3}$, is exposed(56,57). Further, 70 K FN in the absence of FN binds to cells with the same affinity as FN and when present with exogenous FN, 70 K FN blocks FN assembly(6,54,58). Finally, FN assembly and thrombus build-up are reduced by FUD(29,30). Receptors for 70 K FN and whether the receptors are the same on platelets and fibroblasts are not known. Recent work showing that NGR sequences can spontaneously isomerize to integrin binding *iso*DGR sequences led to the hypothesis that NGR sequences in 70 K FN interact with integrins and are responsible for the binding of FN to the cell surface(48). Additionally, IGD sequences, of which there are four in 70 K FN, interact with integrins to stimulate fibroblast migration(59). It has been suggested that these sequences could provide the N-terminal interaction of FN for fibril formation(60). However, we feel that other hypotheses also need to be considered.

Roles of FN in diseases associated with thrombosis

The population studies of FN concentration and coronary artery disease may report remote effects of FN(31-36), but such effects are only beginning to be studied.

Vascular remodeling

Vascular remodeling due to hemodynamic changes or vessel injury causes changes in the architecture of blood vessels and is associated with coronary artery disease and atherosclerosis. Vascular remodeling causes changes in the media/lumen ratio of vessels as well as alterations in matrix synthesis, cell proliferation, and apoptosis(61). Periadventitial administration of FUD to mice after partial ligation of the left internal and external carotid arteries resulted in decreased measures of post-stenotic vascular remodeling including intima-media thickness and FN and

collagen deposition(62). This implicates FN deposition into the extracellular matrix as a key pathologic process in vascular pathophysiology.

Atherosclerosis

Atherosclerosis is associated with an increase in total FN within the arterial wall as well as the appearance of FN containing EIIIA or EIIIB(63). ApoE^{-/-}/FN EDA^{-/-} and ApoE^{-/-}/FN EDA^{+/+} mice have smaller atherosclerotic lesions than ApoE^{-/-} mice with normally spliced FN (4,15). Macrophages from ApoE^{-/-}/FN EDA^{-/-} mice showed decreased uptake of lipoproteins, which lead to decreased foam cell formation(15). This suggests that accelerated atherosclerosis is associated with a lack of the proper mix of FN splice variants. Further, lipid-filled smooth muscle cells lose their ability to assemble FN and type I collagen(64). This could impair the stability of the lesion.

Cardiac repair after myocardial infarct

Repair of injured cardiac tissue after a myocardial infarct is a regulated process in which plasma FN and fibrinogen play an important role in establishing the provisional matrix after the inflammatory phase(65). In mice, knock-out of fibrinogen reduces infarct size. In humans, administration of a naturally occurring peptide that blocks fibrin's interaction with vascular endothelial cadherin did not affect infarct size, but reduced area of necrosis as described in ref (65). We are not aware of any studies specifically targeting FN in this process.

Targeting FN in thrombotic disease

Ideally, drugs targeting thrombosis should not alter normal hemostasis. Though unknowns remain, it is probable that FN contributes to thrombus formation and the diseases associated with thrombus formation without affecting normal hemostasis. The fundamental question of whether altering the role of FN in thrombosis has the ability to decrease thrombotic events remains. Current antiplatelet drugs including eptifibatide, which targets α IIb β 3, do not alter FN assembly by platelets *in vitro*(23). As mentioned above, FUD, a peptide from the F1 adhesin, blocks platelet build-up in *in vitro* flow chamber assays and decreases vascular remodeling after ligation of carotid arteries in mice. Though FUD is unlikely to be clinically useful because of its size, studies with FUD suggests that N-terminal interactions of FN could be a novel target for blocking FN matrix assembly and likewise the treatment of coronary artery disease. Further, it is becoming increasingly clear that inclusion of EIIIA contributes to the prothrombotic potential of FN. Additional studies are needed to clarify origins of EIIIA-containing FN in plasma and the relative contributions of total FN concentration and fraction that contains EIIIA to thrombus formation.

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Figure 1. Drawing of platelet thrombus volume versus concentration in the *in vitro* flow assay

This drawing depicts the relationship between platelet thrombus volume after 5 min of flow of a mixture of platelets, red cells, and FN over a surface coated with FN-fibrin and the concentration of FN used in coating and during the perfusion(29). Arrows labeled (+/+), (+/-), and (-/-) approximate the plasma FN concentration in C57BL/6J mice with two alleles (+/+) of plasma FN, heterozygotes with a knock-out of plasma FN (+/-), and homozygotes for the conditional knock-out of plasma FN (-/-), respectively(38-40). Also located are approximations of the mean values of FN concentration of diseased (solid arrows) and non-diseased subjects (broken arrows) in surveys of plasma FN concentration of patients with coronary disease conducted in China (C)(35), Turkey (T1)(31); (T2)(32); (T3)(34), Korea (K)(33), and United States (US)(36).