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Shear-dependent fibrillogenesis of fibronectin: Impact of platelet integrins and actin cytoskeleton

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ABSTRACT

Soluble plasma fibronectin (Fn) with its inactive compact structure requires unfolding to assemble into active fibrils, which play a role in hemostasis and thrombosis. Fn fibril assembly involves Fn binding to cell receptors, biomechanical coupling of Fn to the cytoskeleton by integrins, exposure of self-assembly sites via contractile cell forces, and elongation of fibrils by Fn polymerization. In this report, we investigated the effect of platelet integrins and actin cytoskeleton on conformational changes of Fn induced by shear. Plasma Fn, in the presence or absence of washed platelets, was exposed to dynamic shear simulating venous or arterial flow conditions. Platelet integrins (αIIbβ3, αvβ3, and α5β1) were blocked by inhibitory antibodies to determine their contribution to shear-induced Fn fibrillogenesis. To examine the role of platelet cytoskeleton in Fn fibrillogenesis induced by shear, platelets were preincubated with cytoskeleton drugs, i. e jasplakinolide to stabilize actin or cytochalasin D to inhibit actin polymerization. Microscopic analyses demonstrated that flow and resulting shear stress over a broad range of physiological and pathological rates (50–5000 s^{-1}) could induce conformational changes of plasma Fn. In addition, the formation of Fn fibrils is modulated by platelet integrins. In this respect, β3 integrins play a dominant role in terms of Fn fibrillogenesis induced by shear. Disruption of the actin polymerization markedly diminished Fn unfolding and assembly. These observations lead to the conclusion that Fn-integrin β3-cytoskeleton interaction is crucial for the assembly of plasma Fn matrix under flow conditions.

1. Introduction

Plasma Fn is synthesized by hepatocytes and secreted into the blood plasma, where it circulates in a concentration of 300–400 μg/ml [1] in a soluble, compact, and inactive form. This dimeric glycoprotein (230–270 kDa subunits) composes of three types of repeating globular modules (FnI, FnII, and FnIII) [2,3]. FnIII1, FnIII2, FnIII7, FnIII10, and FnIII15 domains possess cryptic binding sites which play a crucial role in polymerization and stabilization of assembled Fn [4–8].

Intrinsic functions of Fn *in vivo* are prevalent to multimeric Fn fibrils. Fn unfolding into functional form exposes binding sites buried in the soluble structure to promote the interaction of Fn with other Fn molecules, extracellular matrix (ECM) components, and cell receptors and therefore better support cellular activities [9,10]. Plasma Fn does not form multimeric fibrils spontaneously *in vivo* [11]. The process to incorporate Fn into multimer fibrils can occur both in cell-dependent and cell-free systems. Cell-dependent Fn assembly requires binding of Fn to integrins and subsequent cytoskeletal reinforcement generating tension forces to induce conformational changes of Fn and initiate fibril formation [12]. It has been shown that platelet integrins, αIIbβ3 (glycoprotein IIb/IIIa), αvβ3, and α5β1, could bind to the RGD motif of FnIII10 module [13,14]. Interactions between Fn binding integrins and an intact actin cytoskeleton are critical for the assembly of a Fn matrix [15]. Cell contractility is also required for assembly of Fn into fibrils, and that stretching exposes cryptic sites embedded in the protein [6,9,16,17]. In the cell-free system, Fn fibrils are formed by incubating soluble Fn dimers with anastellin (a Fn fragment which

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induces the formation of Fn multimers), by denaturing soluble Fn with guanidine, or by exposing the Fn molecules to high shear rates [6,8,18]. In the present study, we exposed soluble Fn to flow-simulated shear to determine the effect of platelets integrins and actin cytoskeleton on Fn unfolding upon shear generated by a cone-plate rheometer. We observed that Fn matrix assembly is induced by flow-simulated shear, platelet integrins and cytoskeletal forces.

2. Materials and methods

2.1. Fn purification and labeling

Human plasma Fn was isolated and labeled with alexa fluor 488 succinimidyl ester (AF488) as previously described [10]. Concentrations and corresponding conjugation ratios (dye/Fn molecule) were determined by reading the absorption at 280 nm and 496 nm. The calculation was performed according to the user manual.

2.2. Platelet preparation

Human blood from healthy adult volunteers was collected into acid-citrate-dextrose containers. Whole blood was subjected to centrifugation at 307*g* for 10 min to obtain platelet-rich plasma (PRP). Thereafter, 2 ml PRP were transferred to a plastic tube containing 2 ml PBS, pH 6.5, and 2.5 U/ml Apyrase, followed by centrifugation at 854*g* for 6 min. A platelet pellet was obtained and carefully resuspended in 500 μl of HEPES Tyrode's buffer at room temperature. Platelet number was counted using a hematology analyzer (Sysmex KX-21N, Sysmex, Kobe, Japan).

2.3. Exposure to shear

Polystyrene plates were coated with Fn, collagen (Sigma, St. Louis, USA), or BSA (100 μg/ml) (Sigma, St. Louis, USA), at 37 °C for 1 h and subsequently blocked with 1% BSA. Prior to exposure to shear, 1 ml of Fn in solution at concentration of 100 μg/ml, in the absence or presence of washed platelets (2.5×10^7/ml), was added. Subsequently, the Fn solutions were exposed to shear generated using a cone-plate rheometer (Haaka Rheostress 1; Thermo Scientific, Karlsruhe, Germany). The generated shear rates stepwise increased from 50 to 5000 s^{-1} within 5 min and subsequently decreased from 5000 to 50 s^{-1} within 5 min. The viscosity of Fn solutions was recorded over 10 min. For microscopic analysis (LSM 510, Carl Zeiss, Jena, Germany), a 100 μg/ml of Fn mixture (labeled Fn:unlabeled Fn, 1:10 ratio) was used. In parallel experiments, N-terminal 70kDa fragment of Fn (70 μg/ml) was incubated with Fn mixture at room temperature for 20 min before exposure to shear. Control experiments were conducted at static conditions.

To examine the role of distinct platelet integrins on fibril formation of Fn, washed platelets were incubated with the monoclonal antibodies LM609 (Millipore, Schwalbach, Germany), P1D6 (Abcam, Cambridge, UK), 10E5 (a gift from Barry Coller (Rockefeller University, New York, NY, USA)), or C7E3 (Lilly, Bad Homburg, Germany) (10 μg/ml, each) for 30 min at room temperature to block αvβ3, α5β1, αIIbβ3, or both αIIbβ3 and αvβ3, respectively, prior to the addition of Fn (100 μg/ml) and subsequent shear exposure. To study the effect of cytoskeleton forces on Fn fibrillogenesis, washed platelets were pre-incubated with 1 μM jasplakinolide or 10 μM cytochalasin D (Sigma, St. Louis, USA).

2.4. Western blot analysis of Fn fibril formation

To quantify the amount of fibril formation, (deoxycholate) DOC solubility assay and Western blotting were performed. Shear exposed Fn solutions were removed from plates and precipitated with 2% DOC solution (Sigma, Steinheim, Germany). The DOC-insoluble pellets containing Fn fibrils were isolated by centrifugation at 19,019 *g* for 20 min at 4 °C. The supernatant was removed and saved as the DOC-soluble fraction, whereas the pellet was washed with 2% DOC buffer and resuspended in 1% SDS buffer. For Western blotting, insoluble and soluble Fn were resolved in a 6% SDS-PAGE under reducing conditions and blotted onto nitrocellulose membranes (0.45 μm, Bio-Rad, Germany). The membranes were blocked for 1 h at room temperature using 5% (w/v) nonfat dry milk in PBS, containing 0.1% Tween-20 (PBS-T). The blot was subsequently incubated for 2 h with polyclonal rabbit anti-human Fn antibodies (Abcam, Cambridge, UK), diluted 1:2500. The membranes were washed twice in PBS-T and incubated with HRP-conjugated anti-rabbit antibody (GE Healthcare, Buckinghamshire, UK) at room temperature for 90 min. The blot was washed three times in PBS-T and subsequently incubated with Supersignal West Dura Extended Duration Substrate solution (Thermo Scientific, IL, USA) according to the manufacturer's protocol. Densitometric analysis of immunoreactive bands was conducted using Chemidoc XRS imager and Quantity one software (Thermo Scientific, CA, USA).

2.5. Statistics

All experiments were conducted in at least 3 independent experiments and carried out using blood of different volunteer blood donors. Data were expressed as mean ± SD. One way ANOVA was used for comparison of 2 groups. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Fn unfolding is induced by shear and platelets

Plasma Fn was exposed to dynamic shear (50–5000 s^{-1}). The change in viscosity of Fn was recorded. Consistent with previous finding [18], ultimate viscosities of Fn exposed to shear were significant higher than initial viscosities (Fig. 1). The correlation between increase in viscosity and protein unfolding has been recognized for many years [19,20]. Hence, increase in viscosity upon shear exposure, as depicted in Fig. 1, could indicate fibril formation of Fn. Addition of platelets resulted in a higher increase in viscosities especially on collagen surfaces, where platelets adhere and become activated by the agonist collagen, as compared to samples without platelets.

Microscopic analyses showed that exposing Fn, in the absence of platelets, to shear resulted in fibril formation (Fig. 2D-E), supporting our earlier observations [18]. In this respect, collagen surfaces dramatically enhanced Fn assembly, as compared with Fn surface. This observation is consistent with the data regarding the increase in viscosity of mixture upon shear (Fig. 1). As depicted in Fig. 2C&D, the shear exposure of Fn solutions resulted in fibril matrix in which fibrils linked with one another. Fn fibril diameter varied from 0.5 to 5 μm. Observed fibrils were linked with each other and varied in length. In contrast, short and discrete Fn fibrils were formed on BSA surfaces (images not shown). There were no fibrils observed in the samples at static condition (Fig. 2A). Treatment of Fn with the N-terminal 70 kDa fragment, which is known to inhibit Fn matrix assembly, blocked the fibril formation of Fn exposed to shear (Fig. 2B).

3.2. Fn-binding integrins differentially modulate Fn fibrillogenesis

To determine whether platelet integrins αIIbβ3, αvβ3, and α5β1 cause similar effects on Fn fibrillogenesis induced by shear,

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