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The isolation of fibrinogen monomer dramatically influences fibrin polymerization

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ABSTRACT

Fibrin polymerization begins with the thrombin-catalyzed cleavage of fibrinopeptides from fibrinogen and proceeds through several assembly steps to form an insoluble fibrin clot. Using dynamic light scattering (DLS), we found that purified fibrinogens are polydisperse, containing small amounts of fibrinogen complexes. In order to characterize the impact of these complexes, we used gel filtration chromatography to isolate monomers from three fibrinogens: plasma, recombinant, and recombinant variant Aα251. SDS-PAGE analysis showed that the polypeptides in the monomers were indistinguishable from those in the initial fibrinogen. DLS showed the fibrinogen monomers were monodisperse. We used turbidity to follow polymerization and found the polymerization of fibrinogen monomers was markedly different from the polymerization of the initial fibrinogen; the final optical density (OD) was significantly higher for monomers. Moreover, the polymerization curve for fibrinogen monomers was independent of the polymerization curves of the fibrinogen samples without gel filtration. For example, monomers isolated from two recombinant fibrinogen preparations polymerized similarly even though the final OD increased 2-fold for one preparation and 3-fold for the other. Scanning electron microscopy of the fibrin clots verified the turbidity data; monomer clots had thicker fibers. We conclude that fibrinogen complexes alter the kinetics of polymerization and impair the assembly of monomers into protofibrils and fibers.

Introduction

During blood coagulation soluble fibrinogen is converted to an insoluble fibrin clot (for review including primary references see Weisel [1]). The reaction is initiated by the thrombin-catalyzed cleavage of fibrinopeptides from fibrinogen to generate fibrin monomers. Fibrin monomers then spontaneously polymerize into fibrin fibers. Fibrin polymerization is commonly monitored as the change in turbidity with time after addition of thrombin to fibrinogen. The usual turbidity curve has three phases: a lag phase, which corresponds to the formation of double-stranded protofibrils; a rapid rise in optical density (OD), which corresponds to the lateral aggregation of protofibrils into fibers; and a final OD, which reflects the average size of the fibers and fiber bundles in the clot. Differences in polymerization correlate with differences in fibrin clot structure: clots with thicker fibers have a higher final OD.

We and others have used turbidity and scanning electron microscopy (SEM) to compare polymerization and clot structure of natural and engineered fibrinogen variants. Recently, for example, Allen et al. [2] have compared fibrinogens that are encoded by alternatively spliced mRNAs and Marchi et al. [3] have compared normal plasma fibrinogen to the dysfibrinogen Bβ Y41N. These reports showed differences between the compared fibrinogens and in each case the authors concluded that specific residues influenced polymerization and clot structure. We have used engineered variant fibrinogens as novel reagents to probe the molecular interactions that mediate fibrin clot formation. We have used plasma fibrinogen and normal recombinant fibrinogen as controls and compared data obtained with variant recombinant fibrinogens [4]. The control and experimental samples were run under identical conditions and the differences between these samples were reproducible. We have noted, however, variability in turbidity from experiment to experiment and from one laboratory to another. As it is well-established that environmental factors such as temperature, pH and salt influence polymerization, we presumed this variability arose from differences in reactions conditions [1]. Recently, we monitored fibrin polymerization by dynamic light scattering (DLS) [5]. As DLS measurements are inordinately influenced by high molecular weight species, we removed large material from our fibrinogen preparations by size-exclusion chromatography. This work led us to discover that fibrinogen preparations contain fibrinogen complexes and removal of these complexes led to substantive changes in polymerization.

In the experiments described here we examined three fibrinogens: human plasma fibrinogen, normal recombinant fibrinogen and a recombinant variant fibrinogen Aα251. The results showed that

Abbreviations: DLS, Dynamic light scattering; OD, Optical density; SEM, Scanning electron microscopy.

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polymerization and clot structure were altered when fibrinogen was further purified by gel filtration chromatography. These findings are important to all investigations that examine polymerization and clot structure using purified fibrinogens. Moreover, as high molecular weight complexes containing fibrinogen are present in normal plasma [6], these findings may also be critical for studies with isolated plasma and studies in vivo. Indeed, as clots with thinner fibers correlate with thrombotic disease, plasma levels of fibrinogen complexes may have a causal role in thrombosis.

Materials and methods

Materials

All chemicals were reagent grade and were purchased from Sigma-Aldrich (St. Louis, MO), unless specified otherwise. Human plasma fibrinogen was from Enzyme Research Laboratories, INC. (South Bend, IN); this FIB 1 material contains all the natural plasma fibrinogen variants and is plaminogen free. Cell culture media with normal recombinant and Aα 251 fibrinogens were obtained from the National Cell Culture Center (Biovest International, Minneapolis, MN). Recombinant fibrinogens were purified by immunoaffinity chromatography as described [7].

Preparation of Fibrinogen Monomers

Fibrinogen monomers were prepared by gel filtration chromatography as previously described [5]. Briefly, fibrinogen was concentrated to ~8 mg/mL in 20 mM HEPES, 0.15 M NaCl, pH 7.4 (HBS), filtered with a 0.22 μm GV DURAPORE centrifugal filter, and 100 μl injected into a Superdex-200 column (GE Healthcare, Piscataway, NJ) equilibrated with HBS. Fractions were analyzed by absorbance at 280 nm (Nanodrop 2000, Thermo Scientific,Wilmington, DE), by SDS-PAGE, by immunoblot and by dynamic light scattering (DLS) with the dynamic light scattering Plate Reader (DynaPro™, Waytt Technology, Santa Barbara, CA), as described [5]. Fractions containing fibrinogen monomers were combined and stored at −20 °C. For the recombination experiment two fractions were collected: 1) from the beginning of the rise in absorbance to the beginning of the monomer fraction and 2) the monomer fraction as shown in Fig. 1A. The concentration in each pool was determined by absorbance at 280 nm, aliquots from each pool were combined to mimic the original mixture and the mix was stored at −20 °C.

Polymerization Measured by Turbidity

Polymerization at ambient temperature was monitored by turbidity, essentially as described [7]. Briefly, 50 μL fibrinogen was placed in a microtiter plate (Corning Costar, Corning, New York, USA) well, and the reaction was initiated by the addition of 50 μl of human α-thrombin using a multichannel pipette. The final fibrinogen and thrombin concentrations were 0.6 μM and 0.1 U/mL, respectively, in HBS with 1 mM CaCl2. For plasma and normal recombinant fibrinogens the concentration was determined using the extinction coefficient at 280 nm of 1.51 for 1 mg/mL and 340,000 Da mass; for Aα 251 fibrinogen the concentration was determined using the extinction coefficient at 280 nm of 1.6 for 1 mg/mL and 265,000 Da mass. Polymerization was followed at 350 nm on a SpectraMax340PC microplate reader (Molecular Devices, Sunnyvale, CA, USA). The curves were graphed using Microsoft Excel 2011. Fibrinogens before gel filtration purification were dialyzed overnight at 4 °C in HBS. Fibrinogen monomers and fibrinogen mixtures were thawed at 37 °C and diluted to 1.2 μM in HBS with 1 mM CaCl2, then were incubated at room temperature for 30 min prior to turbidity tests.

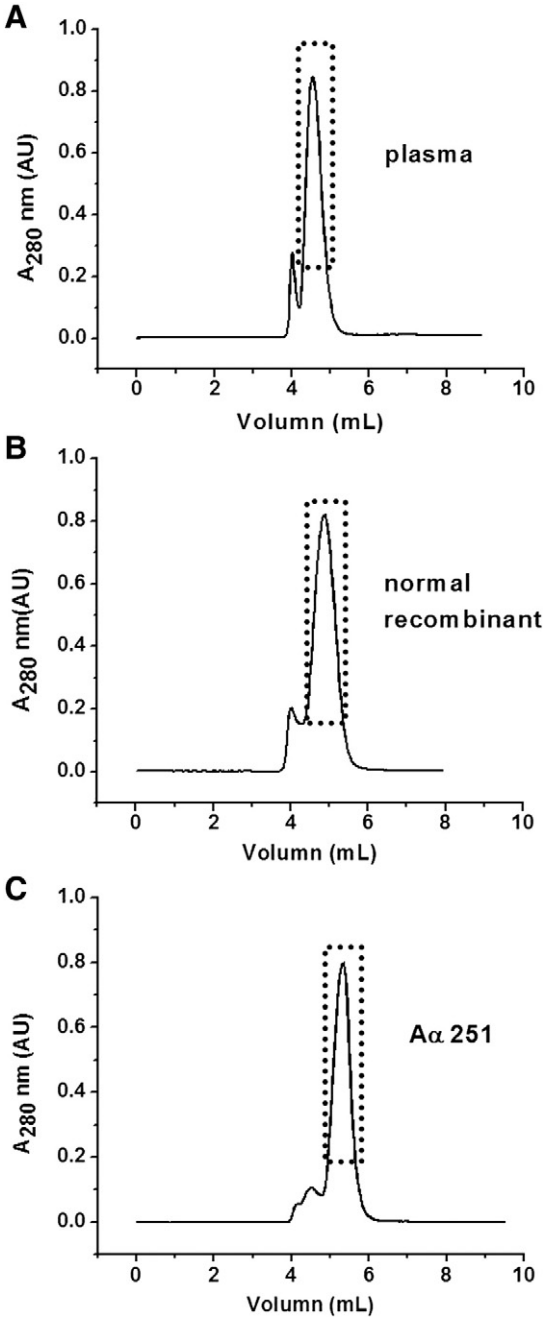

Fig. 1. Preparation of fibrinogen monomers by gel filtration chromatography. One hundred microliters of fibrinogen (~8 mg/ml in HBS) were injected onto a Superdex 200 10/300 GL size-exclusion column at a flow rate of 0.5 mL/min. Elution profiles were monitored at 280 nm. Representative data for plasma fibrinogen (A), high OD normal recombinant fibrinogen (B) and high OD Aα251 fibrinogen (C) are shown. The dashed box shows the monomer fractions that were collected for further analysis.

Scanning Electron Microscopy

Fibrin clots were prepared by mixing 10 μL of 1.8 μM fibrinogen in HBS with 1 mM $CaCl_2$ with 10 μL of 0.2 U/ml of thrombin. This mixture was rapidly transferred to a polycarnonate membrane (Nuclepore Track-Etched Membranes, Whatman). After 24-hr in a moist environment, the clots were processed for scanning electron microscopy by fixation in 2% of glutaraldehyde, serial dehydration in ethanol, critical point drying, and coating as described [8]. Clots were observed and photographed digitally in at least 30 different areas per clot, using a Zeiss Supra 25 scanning electron microscope (Zeiss, Oberkochen, Germany). Experiments were performed twice.