Studies on the Ultrastructure of Fibrin Lacking Fibrinopeptide B (β-Fibrin)

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Release of fibrinopeptide B from fibrinogen by copperhead venom procoagulant enzyme results in a form of fibrin (β-fibrin) with weaker self-aggregation characteristics than the normal product (αβ-fibrin) produced by release of fibrinopeptides A (FPA) and B (FPB) by thrombin. We investigated the ultrastructure of these two types of fibrin as well as that of β-fibrin prepared from fibrinogen Metz (Aα16 Arg→Cys), a homozygous dysfibrinogenemic mutant that does not release FPA. At 14°C and physiologic solvent conditions (0.15 mol/L of NaCl, 0.015 mol/L of Tris buffer pH 7.4), the turbidity (350 nm) of rapidly polymerizing αβ-fibrin (thrombin 1 to 2 U/mL) plateaued in <6 min and formed a “coarse” matrix consisting of anastomosing fiber bundles (mean diameter 92 nm). More slowly polymerizing αβ-fibrin (thrombin 0.01 and 0.001 U/mL) surpassed this turbidity after ≥60 minutes and concomitantly developed a network of thicker fiber bundles (mean diameters 118 and 186 nm, respectively). Such matrices also contained networks of highly branched, twisting, “fine” fibrils (fiber diameters 7 to 30 nm) that are usually characteristic of matrices formed at high ionic strength and pH.

THROMBIN-CATALYZED fibrin formation begins with the release of fibrinopeptide A (FPA) from the amino terminus of the Aα chains of fibrinogen1,5 and is accompanied by the slower release of fibrinopeptide B (FPB) from Bβ chains. Reptilase, a snake venom enzyme from Bothrops atrox, removes only FPA,8 release of which is strictly required for gelation or for lateral aggregation. Thus, although FPB release is not essential for lateral fiber growth, release of fibrinopeptide B from fibrinogen by copperhead venom procoagulant enzyme (CVE)27 is sufficient to cause normal fibrinogen to polymerize.29 Based on their gross appearance, these clots tend to be fine at temperatures below hydrostatic solvent conditions (0.15 mol/L of NaCl, 0.015 mol/L of Tris buffer pH 7.4), the turbidity (350 nm) of rapidly polymerizing αβ-fibrin (thrombin 1 to 2 U/mL) plateaued in <6 min and formed a “coarse” matrix consisting of anastomosing fiber bundles (mean diameter 92 nm). More slowly polymerizing αβ-fibrin (thrombin 0.01 and 0.001 U/mL) surpassed this turbidity after ≥60 minutes and concomitantly developed a network of thicker fiber bundles (mean diameters 118 and 186 nm, respectively). Such matrices also contained networks of highly branched, twisting, “fine” fibrils (fiber diameters 7 to 30 nm) that are usually characteristic of matrices formed at high ionic strength and pH.

Slowly polymerizing β-fibrin, like slowly polymerizing αβ-fibrin, displayed considerable quantities of fine matrix in addition to an underlying thick cable network (mean fiber diameter 135 nm), whereas rapidly polymerizing β-fibrin monomer was comprised almost exclusively of wide, poorly anastomosed, striated cables (mean diameter 212 nm). Metz β-fibrin clots were more fragile than those of normal β-fibrin and were comprised almost entirely of a fine network. Metz fibrin could be induced, however, to form thick fiber bundles (mean diameter 78 nm) in the presence of albumin at a concentration (500 μmol/L) in the physiologic range and resembled a Metz plasma fibrin clot in that regard. The diminished capacity of Metz β-fibrin to form thick fiber bundles may be due to impaired use or occupancy of a polymerization site exposed by FPB release. Our results indicate that twisting fibrils are an inherent structural feature of all forms of assembling fibrin, and suggest that mature β-fibrin or αβ-fibrin clots develop from networks of thin fibrils that have the ability to coalesce to form thicker fiber bundles.

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<25°C and are readily dissociated at 37°C.28,29 Judging from light scattering measurements on fibrin clots formed at 20°C and 37°C, it appears that selective cleavage of FPA at low enzyme concentrations results in the highest branching density and the lowest degree of lateral aggregation.24

In this study, we used electron microscopy to investigate the three-dimensional structure of β-fibrin formed from normal fibrinogen and from the homozygous congenital fibrinogen abnormality known as fibrinogen Metz (Aα Arg 16 → Cys).30,31 To preserve the structures of these fragile fibrin clots, we prepared our material using the critical point drying technique32 as recently used for the study of fine fibrin matrices.14

MATERIALS AND METHODS

Normal fibrinogen fraction I-2 (> 95% coagulable) and fibrinogen Metz fraction I-2 were purified from citrated plasma by the method of Blomback and Blomback.4 In some experiments, chromatographically purified normal peak 1 fibrinogen was used. As assessed by the position of the fibrinogen band in NaDodSO4-polyacrylamide gel electrophoresis (PAGE) (5% acrylamide),33,34 unreduced fibrinogen Metz molecules were devoid of polymeric forms and the preparation was ~95% pure.

Sulphydryl titrations of various fibrinogen preparations were carried out either with Eilman's reagent, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB),35 or with 3H or 14C iodoacetamide (specific activities 140 mCi/mmol and 11.1 mCi/mmol, respectively). After reaction with the iodoacetate, samples were extensively dialyzed, dissolved in 10 mL of Aqueosol-2 liquid scintillation cocktail (New England Nuclear, Boston), and radioactivity was subsequently determined in a Packard model 2425 spectrometer.

Freeze-dried human thrombin (H-I), a gift from Dr D. L. Aronson, was reconstituted in H2O and stored frozen at a concentration of 5,000x to 8,000x to CVE addition to block this activity. Turbidity measurements at 350 nm on fibrin clots were carried out at 14 ± 1°C in a Gilford model 240 spectrophotometer equipped with a jacketed specimen holder.

Certain β-fibrin specimens were subjected to gel-sieving chromatography at 37°C to separate β-fibrin from a minor αβ-fibrin contaminant.36 In brief, following the incubation period with CVE, the clot was overlaid with 1 mmol/L of phenylmethylsulfonylfluoride (PMSF) and incubated in the presence of this reagent for at least 2 hours. The fibrin was then dissociated by being warmed to 37°C, mixed with an equal volume of 0.3 mol/L of NaCl, 0.03 mol/L of Tris buffer pH 7.4 ± 0.1 PMSF 10−4 mol/L, and gel-filtered at 37°C through a Sepharose 2B column equilibrated in this same buffer. Early eluting fractions containing the αβ-fibrin contaminant were discarded, whereas fractions containing “β-fibrin monomer” eluting at a concentration above six-tenths of the β-fibrin peak height were pooled and stored frozen at −30°C at concentrations ranging from 400 to 800 μg/mL. For electron microscopy or turbidity measurements, aliquots of β-fibrin monomer were thawed at 37°C for 30 minutes, diluted 1:1 with H2O, and then incubated at 14°C for the desired amount of time. Preparations were monitored by NaDodSO4-PAGE37 to verify whether anticipated fibrinopeptide cleavages had occurred and to determine if other proteolytic attack had occurred (eg, on Aα chains).

Samples of purified fibrin for negative staining electron microscopy were formed at 14 ± 1°C as 50-μL drops (50 to 500 μg/mL) in a Petri dish that had been humidified with a moist sponge, incubated for 16 to 18 hours with thrombin or CVE, picked up on glow-discharged carbon films, and then negatively contrasted with 2% (wt/vol) uranyl acetate. For processing by the critical point drying technique, clots were picked up on glow-discharged carbon-coated 200-mesh copper grids, fixed with glutaraldehyde (2.5%, vol/vol) in 0.1 mol/L of HEPES buffer pH 7.0, containing 0.2% (wt/vol) tannic acid, washed several times with H2O, stained with 0.5% (wt/vol) uranyl acetate, and washed again with H2O.38 The specimen was then dehydrated in graded ethanol solutions (20% to 100%, vol/vol) and CO2 critical point dried39 using a Tousimis model 780A critical point drying apparatus (Tousimis Scientific Instruments, Rockville, MD).

Plasma fibrin clots were prepared from blood that had been drawn into a plastic syringe, immediately transferred to a centrifuge tube, and then centrifuged at 3,000 g for 3 to 5 minutes to remove cellular elements. The supernatant plasma was incubated overnight at room temperature, and the resulting clot was synerized, washed extensively with saline, and fixed overnight at 4°C in 2% (wt/vol) glutaraldehyde solution in 0.1 mol/L of phosphate buffer pH 7.2. Metz fibrin clots were also prepared without syneresis from purified Metz fibrinogen at 2 mg/mL. The fixed preparation was incubated for 90 minutes with 2% osmium tetroxide in 0.1 mol/L of phosphate buffer, dehydrated in graded ethanol solutions, and embedded in Spurr’s low-viscosity medium.40 Thin sections were prepared using a diamond knife and a Reichert Ultracut ultramicrotome and stained with 5% aqueous uranyl acetate and Sato’s lead citrate solution.41

Electron microscopy was performed with a Philips model 400 transmission electron microscope at 60 to 120 kV or with the University of Wisconsin high-voltage (1,000 kV) electron microscope at Madison, WI. Stereograms were made at magnifications of 5,000x to 22,000X at tilt angles of 3° to 6°.

RESULTS

We prepared β-fibrin at 14°C to minimize cleavage of FPA by CVE,28 and we used this same temperature for polymerization of other forms of fibrin. The standard way of

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processing fibrin for transmission electron microscopy (TEM) is to pick up a clot sample by passing a carbon-coated grid through a fibrin clot. The grid surface is washed subsequently with buffer and uranyl sulfate and then air dried. Samples processed in this way have been suitable for observing the structure of a normal coarse fibrin matrix and for characterizing the structure of certain abnormal fibrins such as fibrinogen Paris I. The Metz clot structure and that of β-fibrin was so fragile, however, that at best we found discontinuous short strands and occasionally anastomosing fiber bundles and thin fibrils adherent to the underlying substrate. To preserve the three-dimensional structure of this type of clot, we processed the sample using the critical point drying procedure. This technique has been used for demonstrating previously unrecognized features of fine clot matrices.

αβ-Fibrin. Images of critical point dried, rapidly polymerizing αβ-fibrin (thrombin, 1 U/mL) formed at 14°C under physiologic conditions (ie, I~0.15, pH 7.4), revealed a coarse matrix consisting predominantly of twisted anastomosing cables (Fig 1A, Table 1). These cables showed typical cross-striations (~22 nm period) when viewed at a higher magnification than that shown in Fig 1 (data not shown).* At a 100-fold lower thrombin concentration (0.01 U/mL), the rate of fibrin polymerization was considerably slowed (Fig 3); nevertheless, the clot ultimately developed considerably greater turbidity than fibrin formed at 1 U/mL. These observations confirm studies indicating that slow rates of fibrin polymerization favor higher degrees of lateral aggregation (ie, thicker fiber bundles). At a thrombin concentration of 0.001 U/mL, clot development proceeded even less rapidly than at 0.01 U/mL although the turbidity eventually exceeded that of rapidly polymerizing αβ-fibrin. NaDodSO4-PAGE analysis of fibrin specimens taken at the time of fixation for critical point drying, indicated that complete FPA release had occurred in all cases, but that in the specimen prepared at a thrombin concentration of 0.001/mL, only 50% of FPB had been released.

Consistent with turbidity measurements, fibrin formed at 0.01 U/mL or 0.001 U/mL revealed a network predominantly composed of large anastomosing fiber bundles that tended to be thicker than those from rapidly polymerized αβ-fibrin (Fig 1). In addition to the thick cable network, however, there was also a prominent network of thinner fibrils. This second type of matrix was most prominent at the lowest thrombin concentration (0.001 U/mL) and consisted of highly branched, twisting fibrils (Fig 4) that appeared to entrap and merge with large fiber bundles. The thick cables forming under this latter condition were composed of loosely packed fibrils (arrows, Fig 1C) and did not display the striations typical of more tightly packed bundles.

β-Fibrin. β-Fibrin gels formed at 14°C can be disassociated by being warmed to 37°C, and the resulting β-fibrin monomer can subsequently be polymerized by incubation at room temperature or below. This process can be carried out repeatedly on material that has been inactivated with PMSF. Turbidity measurements on such a β-fibrin monomer preparation (Fig. 5) indicated that the onset of polymerization...
Table 1. Fiber Diameter of Thick Fiber Bundles of αβ-Fibrin and β-Fibrin

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Concentration (μg/mL)</th>
<th>n*</th>
<th>Fiber Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>αβ-Fibrin (thrombin, 2 U/mL)</td>
<td>50</td>
<td>36</td>
<td>83 ± 37</td>
</tr>
<tr>
<td>αβ-Fibrin (thrombin, 1 U/mL)</td>
<td>500†</td>
<td>89</td>
<td>92 ± 39</td>
</tr>
<tr>
<td>αβ-Fibrin (thrombin, 0.01 U/mL)</td>
<td>500</td>
<td>43</td>
<td>118 ± 59</td>
</tr>
<tr>
<td>αβ-Fibrin (thrombin, 0.001 U/mL)</td>
<td>500</td>
<td>36</td>
<td>186 ± 90</td>
</tr>
<tr>
<td>β-Fibrin monomer</td>
<td>355</td>
<td>55</td>
<td>212 ± 80</td>
</tr>
<tr>
<td>β-Fibrin (CVE, 0.06 U/mL)</td>
<td>500</td>
<td>36</td>
<td>87 ± 30</td>
</tr>
<tr>
<td>β-Fibrin (CVE, 0.13 U/mL)</td>
<td>500</td>
<td>48</td>
<td>135 ± 73</td>
</tr>
<tr>
<td>Metz β-fibrin + albumin + Ca²⁺</td>
<td>500</td>
<td>29</td>
<td>76 ± 32</td>
</tr>
</tbody>
</table>

In matrices containing significant amounts of both "fine" and "coarse" networks, there appeared to be no sharp size transitions in the progression from thin fibers in the fine matrix (7 to 30 nm in diameter) toward thicker fibers approaching the mean values. For purposes of this analysis, we defined the diameter of a coarse fiber bundle as ≥35 nm.

*Number of fibers measured.

†Owing to the thickness of the clot in the critical point dried specimen, it seemed tenuous to assume that areas selected for fiber measurement were representative of the fiber population in the clot. For that reason, we also prepared thin sections of an imbedded specimen under these same conditions. The fiber diameter determined from this type of specimen preparation was 92 ± 26 nm, range 37–150 nm. SD and range of values were lower because we used only the minimum cross-sectional diameter of any given fiber to avoid including measurement of tangential or oblique sections of fibers in our calculations.

Fig 2. Comparison of the ultrastructure of a Metz plasma fibrin clot with Metz fibrin formed in the presence of albumin or Ca²⁺ or both (TEM). (A) Thin section of a normal plasma fibrin clot; (B) thin section of a Metz plasma clot; bar = 500 nm (A and B); (C) critical point dried specimen of Metz fibrin prepared in the presence of 5 mmol/L of CaCl₂; (D) critical point dried specimen of Metz fibrin prepared with albumin 500 μmol/L and CaCl₂ 5 mmol/L; bar = 500 nm (A and B). Arrows (A and B) point to periodic striations that occur in thick fiber bundles.
tion was more rapid than that of $\beta$-fibrin formed by direct addition of CVE (Fig 6). The initial rate of turbidity rise of $\beta$-fibrin forming at the higher CVE concentration (0.66 U/mL), however, was about the same as the value for $\beta$-fibrin monomer extrapolated to a concentration of 500 $\mu$g/mL (ie, 0.061/min v 0.065/min). Electron micrographs of the $\beta$-fibrin monomer clot showed a network of twisting, occasionally anastomosing cables (Fig 7A). The mean fiber diameter was greater than in any other type of $\beta$-fibrin or $\alpha\beta$-fibrin matrix (Table 1).

$\beta$-Fibrin produced by addition of CVE (Fig 7) formed a network of thick cables frequently displaying periodic striations (see footnote, page 1075) typical of $\alpha$-fibrin or $\alpha\beta$-fibrin bundles. The fiber bundles in these networks, like the $\beta$-fibrin monomer network, anastomosed only occasionally relative to $\alpha\beta$-fibrin clots. At the highest CVE level (0.66 U/mL), the matrix revealed occasional areas of fine, fibrillar networks, usually bridging between thick cables (Fig 7B) or appearing to represent an unraveled or frayed portion of a thick fiber bundle. At a lower CVE level (0.13 U/mL), fiber bundles in the matrix were more loosely packed (Fig 7C, arrow) and wider than those formed at the higher CVE concentration. In addition to the predominant thick cable structure, this clot showed a considerable network of fine twisting fibrils (Fig 7C and Fig 4B).

Metz $\beta$-fibrin. Metz $\beta$-fibrin is more transparent, less viscous, and more easily disrupted than normal $\beta$-fibrin. In contrast to normal, Metz $\beta$-fibrin formed by direct addition of thrombin or CVE at 14$^\circ$C or that formed at 14$^\circ$C from Metz $\beta$-fibrin monomer solutions (ie, first incubated with thrombin at 37$^\circ$C), revealed a matrix comprised almost entirely of thin, twisting, and highly branched fibrils (Figs 4C and 7D) that occasionally coalesced to form somewhat thicker, relatively short, uneven bundles. These observations are consistent with the very low turbidity recorded at 350 nm (Fig 6).

To evaluate the type of Metz fibrin matrix formed in vivo, we prepared fibrin directly from native plasma and examined thin sections by electron microscopy (Fig 2). Normal fibrin yielded the expected thick, striated cables (Fig 2A, arrow), whereas Metz fibrin yielded loosely packed fiber bundles that frequently were sufficiently in register to yield a striated pattern (Fig 2B, arrow). Fibrin aggregation is well known to accelerate in plasma, and this is due in part to effects related to the presence of albumin and IgG. The accelerating effect is also accompanied by increased clot turbidity that is attributable, at least in part, to the presence of albumin or IgG, or both, and Ca$^{2+}$. To provide an explanation for the more mature structure of Metz plasma

Fig 3. Turbidity measurements on $\alpha\beta$-fibrin. These experiments were carried out under the same conditions described in Fig 1. Thrombin was added to a final concentration of 1 U/mL, 0.01 U/mL, or 0.001 U/mL; absorbance (A 350 nm) was monitored as a function of time.
additions. 0.66 or 0.13 U/mI, to normal fibrinogen at 14°C were
fl-flbrin was incubated with thrombin (5 U/mL) at 14°C and
several hours; this effect was even more marked when
5 albumin showed increased turbidity developing slowly over
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Metz
made under the same buffer conditions described in Fig 1
+buffer; Metz + CaCl2, 5 mmol/L; Metz + albumin, 500 μmol/L; Metz + albumin, 500 μmol/L + CaCl2, 5 mmol/L.

DISCUSSION
Although many EM studies have been made of the structure of α-fibrin and αβ-fibrin, to our knowledge none have reported on the ultrastructure of β-fibrin. By using the critical point drying technique to process fibrin clots, we were able to preserve clot structure and obtain high-quality, detailed stereo images of β-fibrin and Metz β-fibrin clot networks. Our findings, coupled with turbidometric analyses, permitted a comparison of the structure of αβ-fibrin with that of β-fibrin and Metz β-fibrin.

It is clear from our present findings that release of FPB alone can give rise to clots whose features resemble those of α-fibrin in several important ways: (a) Both types of fibrin tend to form thick twisting cables displaying typical periodic cross-striations under physiologic buffer conditions. When polymerization takes place relatively slowly, both types of fibrin develop fine fibrillar network structures in addition to a relatively thicker cable network. The latter observation is consistent with previous studies indicating that fiber thickness is determined in part by the kinetics of fiber growth.11,12,14-20 (b) The fibrils from both types of fibrin show the characteristic twisting described by Müller and colleagues14 for fine clots. Regions containing fine fibrillar matrix structures, whether derived from normal (β-fibrin or αβ-fibrin) or Metz β-fibrin, are not easily distinguishable from the fine matrix formed by αβ-fibrin at high ionic strength/pH14 or in a slowly polymerizing system under physiologic conditions (this study).

Twisting is not only a characteristic of native fibrin fibrils (ref. 14 and this study), but is also a prominent feature of microcrystals and fibrin clots prepared from enzymatically modified fibrinogen.11,12 We infer from those results and from our present observations that fiber twisting is an
inherent feature of fibrin assembly when polymerization is initiated by release of either FPA or FPB.

Slowly polymerizing αβ-fibrin or β-fibrin networks contain fine fibrillar substructures that are superimposed on and merge with thick, loosely packed fiber bundle networks. Such structures evidently reflect an intermediate phase of fibrin assembly during which thick cables coalesce from a fine fibrillar matrix to form the mature anastomosing cable network. This observation is consistent with the observations that under physiologic conditions mature fibrin assembly develops over a relatively long time from protofibrils that subsequently undergo lateral aggregation to form thick fiber bundles. The precise mechanism by which this assembly process takes place is not yet known.

Clot turbidity is reportedly a function of the second power of fiber diameter. Therefore, such measurements are sensitive to the presence of thick fiber bundles but not to fine fiber networks. The widest cable structures we observed occurred in the coarse β-fibrin monomer clots, yet these gels ultimately developed lower turbidity than did the rapidly polymerizing coarse αβ-fibrin matrix or any other β-fibrin or αβ-fibrin network having lower mean fiber widths (Table 1). This seemingly paradoxical phenomenon may indicate that light scattering per se is not strictly a function of fiber width but may also be related to factors such as tightness of fiber packing, branching, etc. Further studies combining electron microscopy with detailed physical and rheological measurements of clot properties should be most useful in clarifying this issue.

In contrast to turbidity, measurement of clot permeability is a reflection of pore size, coarse clots having greater permeability than fine clots. Shah and colleagues observed that the permeability of fibrin formed at a low thrombin concentration did not rise in proportion to the increase in fiber thickness calculated from turbidity measurements. These observations are consistent with our present demonstration that slow polymerization (αβ-fibrin or β-fibrin) is accompanied not only by increased fiber thickness, but also by the formation of fine fiber networks. Deductions on clot matrix structure based on turbidity and permeability measurements depend on the assumption of a uniform population of network fibers. This is evidently not the case (ref. 17 and this study), especially under circumstances that are intermediate to the extremes of fine and coarse fiber network formation.

Release of FPA and FPB exposes independent binding sites in the NH2-terminal region of the molecule which appear to function cooperatively in the aggregation process. Release of FPA exposes the "A" site, which becomes aligned with the complementary "a" site in the D domain of another molecule. Similarly, FPB release evidently exposes a "B" polymerization site. Efficient utilization of which by the complementary "b" site may depend on proper alignment of two D domains in an αβ-fibrin polymer. Substitution of cysteine for arginine at Aα 16 in fibrinogen Metz evidently impairs this process because Metz β-fibrin, unlike normal β-fibrin, has only a weak tendency for lateral aggregation. This impairment may be due to the existence of a disulfide bridge at Aα 16. The substitution may impose conformational or steric changes that interfere with proper occupancy or orientation of the polymerization site unmasked by release of FPB, thus impairing optimal alignment of fibrils into bundles. Further study of this impairment may lead to new insights into the exact location of the region involved in the ability of fibrin to form lateral associations with other fibrin molecules.
In contrast to purified Metz fibrin, the Metz plasma clot was less fragile, could be synerized, and showed a considerable degree of lateral aggregation, even to the extent of forming easily discernible periodic striations (Fig 7). This "improvement" in Metz clot structure is not surprising since fibrin aggregation in plasma is associated with increased clot turbidity and increased lateral fiber aggregation. When Metz fibrin was formed at concentrations approaching that in plasma or at an albumin concentration in the physiological range (500 μmol/L), we were able to induce an increased degree of lateral fiber association. This effect was even more pronounced when Ca²⁺ was also present. We believe that the range (500 μmol/L), we were able to induce an increased conversion. Adv Prot Chem 27:1, 1973

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Studies on the ultrastructure of fibrin lacking fibrinopeptide B (beta-fibrin)

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