Evidence for a Second Type of Fibril Branch Point in Fibrin Polymer Networks, the Trimolecular Junction

By Michael W. Mosesson, James P. DiOrio, Kevin R. Siebenlist, Joseph S. Wall, and James F. Hainfeld

Fibrin molecules polymerize to double-stranded fibrils by intermolecular end-to-middle domain pairing of complementary polymerization sites, accompanied by fibril branching to form a clot network. Mass/length measurements on scanning transmission electron microscopic images of fibrils comprising branch points showed two types of junctions. Tetramolecular junctions occur when two fibrils converge, creating a third branch with twice the mass/length of its constituents. Newly recognized trimolecular junctions have three fibril branches of equal mass/length, and occur when an extraneous fibrin molecule initiates branching in a propagating fibril by bridging across two unpaired complementary polymerization sites. When trimolecular junctions predominate, clots exhibit nearly perfect elasticity.

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MATERIALS AND METHODS

Formation of fine clots. Human plasma fibrinogen fractions 1-2 and 1-9 were prepared as previously described.29-32 Clots for TEM examination were formed as 50-μL droplets on Parafilm (Amer. Con Co, Greenwich, CT) from fibrinogen fraction 1-2 (25 μg/mL) in 400 mmol/L NaCl, 10 mmol/L HEPES, 5 mmol/L EDTA, pH 8.5 buffer (I = 0.3), or from fibrinogen fraction 1-9 (25 μg/mL) in 290 mmol/L NaCl, 10 mmol/L HEPES, pH 7.0 buffer (I = 0.3), at an α-thrombin concentration of 1 U/mL, and incubated for 2 hours at room temperature. Grid specimens were picked up on ultrathin rotary shadowing with platinum-carbon, and were then examined in a Philips 400 electron microscope (Philips Instruments Co, Rochester, NY).

For TEM experiments, fibrinogen fraction 1-2 or fraction 1-9 solutions were dialyzed against 400 mmol/L NaCl, 10 mmol/L HEPES, 5 mmol/L EDTA, or 1 mmol/L CaCl₂, pH 8.5 buffer (I = 0.4), deposited as 50-μL droplets (16 to 50 pg/mL) on Parafilm, and converted to fibrin at an α-thrombin concentration of 1 U/mL. After incubation of clotting mixtures at room temperature for 60 to 150 minutes, grid specimens were obtained by passing the fibrin

From the Sinai Samaritan Medical Center, University of Wisconsin Medical School, Milwaukee Clinical Campus, Milwaukee, WI; and the Biology Department, Brookhaven National Laboratory, Upton, NY.

Submitted November 30, 1992; accepted April 30, 1993.

Supported by National Institutes of Health Grants No. HL-28444, HL-47000, and RR-01777 (to the Brookhaven National Biomedical STEM Resource Facility).

Address reprint requests to Michael W. Mosesson, MD, Sinai Samaritan Medical Center, 950 N 12 St, Milwaukee, WI 53233.

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0006-4971/93/8205-0017$3.00/0

Blood, Vol 82, No 5 (September 1), 1993: pp 1517-1521

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droplet back and forth several times through a pipette tip before injecting a sample into a droplet of buffer on the grid surface or by passing the grid directly through the fibrin droplet. After 1 minute, the specimen on the grid was washed 8 to 10 times with 50 mmol/L ammonium acetate solution, quickly frozen in liquid nitrogen, freeze-dried, transferred under vacuum to the microscope stage, and imaged by using a 40-kV probe focused at 0.25 nm.

Mass measurements on fibrin fibrils. Mass determinations on STEM images were based on electron scattering measurements and were performed off-line as previously described. A “fiber” program was used for estimating the mass of trimolecular complexes composed of two D and one E domain (D·E·D) complexes within fibrils, or a “user-defined boundary” program for measuring the mass at a fibril branch junction. Tobacco mosaic virus particles were used as a mass calibration standard. The predicted mass/length of fibrils. Plasma subfractions containing mostly intact fibrinogen molecules (ie, fraction I-2) have a mean molecular weight of 340 ± 15 × 10^3, whereas subfractions containing molecules from which COOH-terminal portions of Αα chains have been cleaved (eg, fraction I-9) have a mean molecular weight of 300 ± 15 × 10^3. The mean molecular weight of fraction I-2 fibrin molecules is 334 × 10^3, about 2% lower than the precursor fibrinogen due to loss of both fibrinopeptides; that of fraction I-9 fibrin is 294 × 10^3. The predicted mass/length of double- and four-stranded fibrin fibrils is calculated using the values for fibrin monomer and an assumed length of 46 nm for a fibrin molecule.

RESULTS AND DISCUSSION

Rotary-shadowed TEM images of fine clot networks formed from intact fibrinogen molecules (fraction I-2) or from fibrinogen molecules lacking carboxy terminal regions of Αα chains (fraction I-9) (not shown) showed a highly branched fibril network (Fig 2). Most branch junctions, as judged from fibril widths, were trimolecular branch points (arrows), although tetramolecular branch points were also found (double-shaft arrows) at a lower frequency. Thus, these observations indicate that trimolecular branches are the predominant junctional components in fine clot matrices. Multi-stranded fibers were found commonly (Fig 2B), although they were not as thick or as predominant as in coarse clots. These fibers often possessed protruding thin fibrils forming an arm of a branch junction (arrowheads), but because the fibers were multistranded, the origin of such fibrils as an element of either a trimolecular or tetramolecular branch point could not be ascertained.

STEM images of unstained freeze-dried fine fibrin network fibers showed many fibrils and frequent branches (Fig 3). Fibrils with widths corresponding to double-stranded fibrils that had been formed from intact fibrinogen molecules had mass/length values of 14.4 Kd/nm (EDTA-containing buffer) and 14.8 Kd/nm (CaCl₂-containing buffer) (Table 1), respectively, both values corresponding closely to the
predicted value of 14.5 Kd/nm. As had been observed in rotary-shadowed images, these fibrils were characterized by the presence of oblong to triangular dense structures, 8 to 16 nm across and 19 ± 2.7 nm in length along long fibril axes, regularly spaced ~22.5 nm apart; this interval corresponds to one-half the length of a fibrinogen molecule, and reflects the half-staggered arrangement of fibrin molecules within the fibril. The mass of these structures (eg, the circle in Fig 3A), was 300 ± 38 Kd (integration radius \( r_i \) = 12 nm; \( n = 48 \)), a value corresponding to a D·E·D complex.1 The apices of consecutive triangular-shaped D·E·D complexes commonly were reversed along the fibril (connected arrows, Fig 3A, B, and D), consistent with the fibril twisting that is known to take place.16,17,28 A schematic drawing of these fibrils is presented in Fig 4.

Mass measurements at branch junctions containing a fibril with twice the width of its two constituent thin fibrils indicated that the wider fibril also had twice the mass/length, thus identifying them as tetramolecular junctions (double-shafted arrows, Fig 3A and D). Similarly, junctions consisting of three thin fibrils of equal width and equal mass/length (Table 1) could be unambiguously identified by either criterion as trimolecular junctions (arrows, Fig 3B, C, E). In addition, the mass at the nidus of trimolecular junctions (eg, irregular shape, Fig 3E) was 898 ± 139 Kd (\( n = 7 \)), a value corresponding to the three D·E·D complexes that constitute such a junction (cf, Fig 4). Taken together, these observations indicate that trimolecular branches are initiated by extension of the D domain of a fibrin molecule away from a propagating fibril at an incipient branch junction, concomitant with bridging across the unpaired complementary polymerization sites by an extraneous fibrin molecule, as originally suggested somewhat less concisely.28 To our knowledge, this mechanism of branching has not yet been observed in other types of biologic and synthetic polymer networks.45 It is also interesting to note that this type of branching was recently proposed as a feature of early fibrin polymerization, in the context of an intermediate mechanism for subsequent lateral fibril associations or for unordered fibrin aggregation.46

Fibrin from fibrinogen fraction I-9 molecules have a diminished tendency to form thick fibers,29,30 consistent with evidence suggesting that a second polymerization site (“b”) contributing to lateral fibril association is located in the carboxy-terminal segments of A\( \alpha \) chains.33 This fibrin formed a fine matrix consisting predominantly of trimolecular branch structures (not shown), but at a lower pH (7.0) and ionic strength (0.3) than did intact fibrin. STEM mass measurements of fibril D·E·D nodules yielded a value of 280 ± 39 Kd (\( r_i = 12 \) nm; \( n = 39 \)). The lower mass for these complexes, compared with those in fraction I-2 fibrin (\( P < .02 \)), corresponds to the lower mass found in the vicinity of the E domains of fraction I-9 molecules due to absence of carboxyl-terminal portions of A\( \alpha \) chains.3 Mean thin fibril widths were the same as for fraction I-2 fibrin, as was the 22.5 nm spacing of D·E·D nodules. Their mass/length value (13.8 Kd/nm) was lower than that of intact fibrin fibrils (\( P < .02 \)) and close to the predicted value of 12.8 Kd/nm for this type of fibrin. Similarly, four-stranded fibrils had mass/length values twice that of double-stranded fibrils (Table 1).

The finding that each of the three fibrils constituting tri-

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**Table 1. Fibril Mass/Length**

<table>
<thead>
<tr>
<th>Buffer Condition</th>
<th>Fibrinogen Preparation</th>
<th>Double-Stranded Fibrils</th>
<th>Trimeric Branch Fibrils</th>
<th>Four-Stranded Fibrils</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4 mol/L NaCl, 10 mmol/L HEPES, 5 mmol/l EDTA, pH 8.5 (( l = 0.4 ))</td>
<td>Fraction I-2* (( n = 73 ))</td>
<td>14.4 ± 1.5 (( n = 12 ))</td>
<td>14.9 ± 1.2 (( n = 15 ))</td>
<td>26.4 ± 2.3 (( n = 8 ))</td>
</tr>
<tr>
<td>0.4 mol/L NaCl, 10 mmol/L HEPES, 1 mmol/L CaCl(_2), pH 8.5 (( l = 0.4 ))</td>
<td>Fraction I-2* (( n = 38 ))</td>
<td>14.8 ± 2.1 (( n = 15 ))</td>
<td>14.8 ± 2.3 (( n = 8 ))</td>
<td>29.9 ± 4.2</td>
</tr>
<tr>
<td>0.29 mol/L NaCl, 10 mmol/L HEPES, pH 7.0 (( l = 0.3 ))</td>
<td>Fraction I-9† (( n = 112 ))</td>
<td>13.8 ± 1.6 (( n = 14 ))</td>
<td>13.1 ± 1.4 (( n = 8 ))</td>
<td>24.2 ± 3.4</td>
</tr>
</tbody>
</table>

Values are in kilodaltons per nanometer.

* Predicted mass/length for double-stranded fibril, 14.5 Kd/nm; four-stranded fibril, 29.0 Kd/nm.
† Predicted mass/length for double-stranded fibril, 12.8 Kd/nm; four-stranded fibril, 25.6 Kd/nm.
Fig 4. An illustrated model of fibrin fibrils and fibril branch junctions. Fibrin molecules are represented by a small black oval (central E domain) and two larger ovals (outer D domains) that are connected through the respective E domain by either a solid or dashed line. The D domains of each molecule have the same fill pattern. Fibril assembly occurs by end-to-middle domain interactions between complementary polymerization sites in the E ("A" site) and D domains ("a" site), forming double-stranded, twisting fibrils. Branching occurs at tetramolecular (1) or trimolecular junctions (2). The three D-E-D complexes comprising the nidus of a trimolecular junction are enclosed by a dashed circle. An incipient trimolecular fibril junction is depicted in the lower part of the diagram.

molecular branch points was double-stranded by mass/length criteria, corroborates previous conclusions based on fibril width measurements, provides proof of the existence of these junctions as structural elements of the fibrin network, and is a means for distinguishing them unambiguously from tetramolecular junctions. In view of the close correspondence between fibril widths and mass/length measurements, we conclude that fibril width measurements alone are sufficient for identifying fibril branch junctions. Using this criterion, we note that negatively contrasted TEM images reported by Hantgan et al. and critical point dried TEM images reported by Müller et al. and Mosesson et al. present a number of excellent examples of trimolecular branch junctions formed under coarse or fine clot buffer conditions. In contrast, the number of thin fibrils comprising multistranded fibers can be accurately determined only by STEM mass measurements, because as yet there is no simple correlation between fiber width and the number of constituent fibrils.

It is likely that trimolecular branch junctions play an important role in determining the rheologic properties of fibrin clots that help to define their physiologic function in relation to other clot elements. Lateral fibril associations vastly predominate in coarse clots in which thick fiber bundles are the rule, a composition that evidently accounts for fiber or junctional slippage and realignments that occur in coarse clot networks under stress conditions leading to irrecoverable deformation. On the other hand, trimolecular branch points predominate in fine clot networks, which exhibit nearly perfect elasticity, a property that we attribute to their presence. Their contributions to the properties of coarse clot networks in relation to the other elements of network structure remain to be explored.

ACKNOWLEDGMENT

We thank William Semrad and Karen Mickey Higgins for graphics/photography services.

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Evidence for a second type of fibril branch point in fibrin polymer networks, the trimolecular junction

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