Evaluation of the Role of In Vivo Proteolysis (Fibrinogenolysis) in Prolonging the Thrombin Time of Human Umbilical Cord Fibrinogen

By Dennis K. Galanakis and Michael W. Mosesson

These studies have been directed at evaluating the role played by proteolysis (fibrinogenolysis) in vivo in prolonging the thrombin time of human umbilical cord ("fetal") fibrinogen. The aggregation rate of cord fibrin compared with that from adult plasma is always delayed when the reaction is carried out under conditions of relatively high ionic strength (e.g., 0.29); this difference is not apparent at relatively low ionic strength (e.g., 0.09). In addition, as assessed by turbidimetric techniques, the maximum absorbance attained by cord fibrin is considerably less than that attained by adult fibrin. Coagulable fibrinogen catabolites (i.e., fraction I-5) are present in cord plasma and, like their counterparts from adult plasma, lack various portions of the COOH-terminal region of the Aα chain. However, their presence in plasma does not explain the behavioral differences between cord and adult fibrin. Moreover, differences revealed by turbidimetric comparison of cord and adult fibrin from plasma fraction I-2 persist in fibrin from fraction I-5; it therefore appears that the COOH-terminal region of the Aα chain does not contain the structure(s) accounting for the unique behavior of "fetal" fibrinogen.

COMPARISONS of fibrinogen from umbilical cord ("fetal") and adult human plasma have been the subject of numerous studies during the past 25 yr. The first suggestion that a fetal form of fibrinogen might exist was based upon the observation that the thrombin time of cord plasma was uniformly prolonged with respect to that of adult plasma. Although most studies have confirmed this observation, not all investigators have agreed as to its cause. That is, it is not yet clear whether the difference heralded by thrombin time prolongation reflects fibrinogen production directed by a fetal gene(s) (analogous to fetal and adult hemoglobin), or if this difference reflects post-synthetic modification. The latter possibility is a particularly important one since under normal circumstances in vivo proteolytic attack on native adult plasma fibrinogen is known to result in formation of slowly coagulable catabolic derivatives. On the one hand, von Felten and Straub and Gmürr et al. reported that the thrombin time of plasma samples collected in the presence of Kunitz' pancreatic trypsin inhibitor (KPTI) was normal but those collected without this inhibitor had prolonged thrombin times; in addition, these samples manifested increased levels of serum fibrinogen–fibrin related antigen. For these reasons, they concluded that proteolysis in vitro could account for the observed differences between cord and adult plasma. On the other hand, Krause...
and Maus\textsuperscript{17} and Teger-Nilsson and Ekelund\textsuperscript{13} found uniform thrombin and Reptilase time prolongation in surveys of single donor cord samples collected either in the presence of KPTI\textsuperscript{17} or epsilon-aminocaproic acid.\textsuperscript{13} They therefore suggested that the findings indicated the presence of a qualitatively distinct fetal fibrinogen.

On the basis of available information, it seems reasonable to propose that differences in solvent conditions used for the thrombin time assay might account for some of the discrepant observations. For example, Teger-Nilsson and Ekelund\textsuperscript{13} attributed their inability to show prolongation of the thrombin time of purified cord fibrinogen to this. Guillin and Ménaché\textsuperscript{12} examined the aggregation rate of cord fibrin and found it to be delayed at ionic strengths in the range 0.15–0.29, but not at 0.05.

Although present evidence strongly favors the existence of a qualitatively distinct fetal fibrinogen, it does not yet permit an unequivocal conclusion as to its cause. Our studies on cord fibrinogen were initiated to provide additional insights on this subject by relating structural and functional differences. The present investigation has focused on the role that proteolytic modification in vivo (fibrinogenolysis) may play.

**MATERIALS AND METHODS**

Two blood collection procedures were employed. Cord blood from spontaneous vaginal deliveries was collected from the placental end of the transected cord (prior to removal of the placenta from the uterus) directly into 3.8\% citrate-containing Vacutainer tubes (Beckton, Dickinson, Co., Rutherford, N.J.). Alternatively, 25–49 ml of cord blood from cesarean sections (after removal of the placenta) or spontaneous deliveries was collected into a 50-mI polycarbonate tube containing 1 ml of 19\% Na citrate and 5000 units of KPTI (Trasylol, FBA Pharmaceuticals, New York, N.Y.); heparin (200 units) was also included on particular occasions to minimize the tendency for clot formation. Care was taken to collect blood samples only after blotting the severed end of the cord and discarding the first few milliliters of blood. Any specimen developing a visible clot was discarded. Adult blood was collected by venipuncture using the same anticoagulant mixtures. The fibrinogen level in plasma was determined by the method of Ratnoff and Menzie;\textsuperscript{23} for heparin-containing plasma, Reptilase (obtained from the venom of Bothrops atrox, Pentapharm Ltd., Basel, Switzerland) instead of thrombin was used to produce a clot.

Plasma was fractionated by a modification\textsuperscript{21} of the glycine precipitation procedure of Kazal et al.\textsuperscript{24} to obtain fractions I-2 and I-5. Fibrinogen concentrations were estimated from absorbance measurements. An absorbance coefficient (A\textsubscript{\text{nm}}) of 15.5 at 280 nm\textsuperscript{11} was assumed. The thrombin clottability\textsuperscript{21} of cord or adult fraction I-2 was 92\%–98\%; that of fraction I-5, 85\%–95\%; this range of clottability was also obtained for fibrinogen fractions prepared from heparin-containing plasma.

The initial glycine step for citrated plasma samples resulted in precipitation of 90\%–93\% of adult (six plasma pools) and 82\%–91\% of cord (five plasma pools) fibrinogen. For heparin-containing plasma the amount precipitated was 94\%–96\% for cord (three plasma pools) or adult (two plasma pools) plasma samples. There were observable differences in the yield of fraction I-2 from adult plasma (35\%–40\%) compared with that from cord plasma (40\%–48\%), but these were not further investigated. The yield of fraction I-5 was the same (3\%–7\%) for both cord and adult plasma pools. The presence or absence of KPTI during blood collection and during subsequent fractionation (i.e., 100–200 U/ml) did not affect the yield.

Thrombin times of plasma fractions were measured with a Fibrometer (BBL, Cockeysville, Md.) at 37\°C, by adding thrombin (0.1 ml, 3 U/ml) in 0.01 M Tris–HCl or 0.01 M sodium phosphate buffer, pH 7, to a solution of fibrinogen (0.1 ml, 3 mg/ml) in the same buffer. In addition, the final clotting mixture also contained NaCl which was added as necessary to obtain a desired ionic strength (the exact ionic strengths are indicated in the results section). The thrombin used was
of human (lot H-1, provided by Dr. D.L. Aronson, Bureau of Biologics, FDA) or bovine (Thrombin Topical, Parke-Davis Co., Detroit, Mich.) origin. The Reptilase time was carried out by diluting the contents of one vial of Reptilase (the salt content of this sample was not specified) with 5 ml of 0.01 M sodium phosphate–0.27 M NaCl buffer (ionic strength at least 0.28), pH 7, and adding 0.1 ml of this solution to 0.1 ml fibrinogen solution (diluted in the same buffer). The fibrinogen–fibrin conversion was also monitored optically after addition of Ancrod or human thrombin using the same solvents employed for thrombin time determination; the highly purified snake venom enzyme was obtained from *Ankylodon rhodostoma*, and was a gift from Grant H. Barlow, Abbott Laboratories.

The aggregation rate of "fibrin monomer" solutions was measured optically by a modification of the turbidimetric method of Donnelly et al as follows. Fibrin clots obtained after addition of human thrombin (0.5–1 U/ml) were extensively washed, synerized and then dissolved in 1 M NaBr (pH 5.3) to a concentration of 7.5–9.8 mg/ml. To 1 volume of fibrin monomer solution, 14 volumes of sodium phosphate buffer (0.1 M, pH 6.35) were added at room temperature, and the absorbance monitored at 350 nm.

S-sulfo derivatives of fibrinogen were prepared according to the method of Pechère. Separation of the S-sulfo fibrinogen chains was achieved by CM-cellulose gradient elution chromatography (Whatman CM 23) as previously described.

Noncross-linked fibrin clots were obtained from plasma to which had been added EDTA (10 mM, final concentration) before tenfold dilution with 0.15 M NaCl and 0.01 M sodium phosphate buffer, pH 7, containing KPT (100 U/ml). Human thrombin was then added (0.5 U/ml, final) and clots that formed were synerized, washed several times with the diluting buffer and finally with distilled water. Fibrin cross-linking experiments on plasma subfractions and subsequent sample reduction for electrophoresis were performed under the conditions outlined by Finlayson et al.

Dodecyl sulfate polyacrylamide gel electrophoresis was performed by the technique of Weber and Osborn. Dithiothreitol was used for reduction of disulfide bridges. Functional identification of fibrinogen chains containing peptides A or B was made in this electrophoretic system after thrombin or Reptilase treatment. Densitometric scanning of gels was performed with a gel scanning device produced by Helena Laboratories (Beaumont, Tex.) and equipped with an integrator. Alkaline polyacrylamide electrophoresis in a 3-mm gel slab containing 8 M urea was carried out in Tris–EDTA–borate buffer, pH 8.6. Acidic polyacrylamide gel electrophoresis was carried out according to Brummel and Montgomery.

### RESULTS

#### Studies on the Fibrinogen–Fibrin Conversion

Studies were carried out to define solvent conditions for revealing functional differences between adult and cord fibrinogen. Thrombin times of fraction 1-2 were examined at pH 7–9 (Table 1), at fibrinogen concentrations of 0.2–2 mg/ml, and at ionic strengths of 0.06–0.30. The most critical of these variables proved to be the ionic strength (Fig. 1). Under conditions of relatively high ionic strength (0.29), fibrinogen from pooled cord plasma (four preparations) always exhibited a prolonged thrombin time (bovine or human thrombin). At a

<table>
<thead>
<tr>
<th>pH</th>
<th>Adult (sec)</th>
<th>Cord (sec)</th>
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<tr>
<td>7</td>
<td>34</td>
<td>61</td>
</tr>
<tr>
<td>8</td>
<td>33</td>
<td>56</td>
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<tr>
<td>9</td>
<td>36</td>
<td>60</td>
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Thrombin times of adult and cord fibrinogen (fraction 1-2, 2 mg/ml) in Tris–HCl buffer at pH's of 7, 8, and 9. The values reflect the mean of at least three determinations. The range was ± 2 sec for adult fibrinogen and ± 4 sec for cord fibrinogen. Depending upon the pH, the ionic strength was between 0.28 and 0.30.
fibrinogen level of 0.7 mg/ml or less, the difference between adult and cord fibrinogen was even more pronounced. The magnitude of this difference was relatively constant between pH 7 and 9 (Table 1). The Reptilase times of cord and adult fibrinogen were also compared at a relatively high ionic strength (fibrinogen concentration, 1.5 mg/ml). Under these conditions (results not shown) the clotting times for adult fibrinogen ranged from 30 to 34 sec, while those of cord fibrinogen were between 53 and 58 sec.

The clotting time difference between cord and adult fibrinogen was also examined turbidimetrically after adding Ancrod (a thrombin-like enzyme which cleaves only peptide A33–34) by monitoring the absorbance at 350 nm (Fig. 2). The higher the ionic strength, the more delayed the onset of clot formation (i.e., increase in absorbance) and the lower the maximum absorbance reached. At an
ionic strength of 0.09 no significant differences between adult and cord fibrinogen were evident. At an ionic strength of 0.17 cord fibrinogen displayed both a slower aggregation rate and a lower maximum absorbance. These differences became even more marked at an ionic strength of 0.29. When thrombin (0.3 U/ml) was substituted for Ancrod at this ionic strength (results not shown) the findings were substantially the same, except that thrombin-treated samples developed higher maximum absorbance than corresponding Ancrod-treated samples.

Further investigation of the nature of the delayed thrombin time was carried out by turbidimetric comparison of the aggregation rates of fibrin monomer solutions prepared from cord or adult plasma fraction 1-2 and 1-5 (Fig. 3). Adult fraction 1-2 fibrin showed an immediate onset of aggregation and the most rapid rise in absorbance. The absorbance of cord fraction 1-2 fibrin also began to rise immediately following dilution of the monomer solution with phosphate buffer, but rose more slowly and attained a lower maximum absorbance. Fibrin from adult fraction 1-5 is enriched with coagulable catabolite species of fibrinogen that manifest a long thrombin time as a result of proteolytic attack in vivo on Aα chains. This monomer preparation showed the expected delayed onset of aggregation, and a slower rate of aggregation than did fraction 1-2 fibrin. The ultimate absorbance reached, however, was the same for both adult fibrin preparations. Cord fraction 1-5 fibrin also displayed a delayed onset of aggregation and had an even slower rate of aggregation than its adult counterpart. The maximum absorbance reached was much lower than that attained by adult fraction 1-5. (The basis for the structural implications of this finding is developed further in the following section and in the discussion section.) Cord and adult fibrin cross-linking (fraction 1-2) was also examined. No differences were evident in the rate or in the ultimate degree of cross-link formation (i.e., γ-dimers and α-polymers), as assessed by dodecyl sulfate gel electrophoresis of reduced samples taken during the course of the experiment.

Fig. 3. Aggregation rates of cord and adult fibrin monomer preparations obtained from fractions 1-2 or 1-5. The reaction was monitored by absorbance measurement at 350 nm. The final concentration of fibrin was 0.65 mg/ml in the case of cord fraction 1-5 and 0.5 mg/ml for all other samples. The abscissa indicates the time after dilution of fibrin monomer solution with sodium phosphate buffer, pH 6.35 (ionic strength 0.18). At the completion of each experiment fibrin clots were synerized and removed; the amount of residual protein in the clot liquor, as assessed by absorbance measurements, was less than 1%.
Evaluation of Cord Fibrinogen Structure

Further comparison of adult and fetal fibrinogen was made by examination of their subunit chain structures. In one series of experiments, S-sulfo derivatives of cord and adult fraction I-2 were prepared and the chains subjected to CM-cellulose gradient elution chromatography. No differences in the elution profile of adult (four preparations) and cord (five preparations) samples were observed. The peaks representing the Aα, Bβ, and γ chains were pooled and subjected to polyacrylamide gel electrophoresis under alkaline urea or acidic urea conditions, or in the presence of sodium dodecyl sulfate; no differences

<table>
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<th>Electrophoretic Band</th>
<th>Adult (Total)</th>
<th>Cord (Total)</th>
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<tbody>
<tr>
<td>Aα + Aα/2</td>
<td>57.6</td>
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<td>Aα/4</td>
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</tr>
<tr>
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<td>3.8</td>
<td>3.9</td>
</tr>
<tr>
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<td>Total</td>
<td>100%</td>
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The relative distribution of Aα chains and the various Aα/ remnant chains assessed by densitometry of dodecyl sulfate gels (9% acrylamide) of S-sulfo Aα chains from adult or cord fraction I-2. The gels were stained with Coomassie brilliant blue.

Fig. 4. Sodium dodecyl sulfate gel electrophoresis (9% acrylamide) of reduced fibrinogen fraction I-5 from citrated cord or adult plasma. Since these gels were from different electrophoretic experiments, the bands are aligned with respect to the Bβ and γ chains, rather than with respect to their origins. The nomenclature for designating the Aα chain remnants lacking various portions of the COOH-terminal region (i.e., Aα/2, Aα/4, etc.) is the same as previously used.
IN VIVO PROTEOLYSIS

Fig. 5. Sodium dodecyl sulfate gel electrophoresis (9% acrylamide) of reduced noncross-linked fibrin clots obtained from adult plasma (gel 1) or from cord plasma from spontaneous deliveries (gel 2) or cesarean sections (gel 3). Plasma samples were collected and clotted in the presence of KPTI. Relatively heavy sample loads are shown in order to clearly display the presence of α/ remnants.

between cord and adult fibrinogen chains were observed. Evaluation of the presence or absence of peptides A or B on isolated S-sulfo chains was conducted by determining their migration rates in sodium dodecyl sulfate gels before and after treatment with thrombin or Reptilase. Comparison by this technique also failed to disclose any differences; i.e., like their adult counterparts, cord Bβ and γ chains were indistinguishable from those in adult fibrinogen, and Aα chains exhibited all remnants (Aα/ previously identified in adult Aα chain preparations.29 Densitometric scanning of gels permitted quantitative assessment of the distribution of Aα chains and Aα/ remnants (Table 2), and revealed no significant differences.

Cord fraction 1-5, like adult fraction 1-5, contains a much higher proportion of Aα/ remnants than does fraction 1-2. Consistent with the findings for the S-sulfo chains of fraction 1-2, when reduced samples of adult and cord fraction 1-5 were compared (Fig. 4), no differences in the distribution of Aα/ remnants were observed. Fraction 1-5 having the characteristics shown in Fig. 4 was obtained from adult or cord plasma whether the sample was collected into KPTI or not and whether or not fractionation was carried out in the presence of KPTI. Furthermore, fibrin clots obtained from cord plasma always showed the presence of α/ remnants regardless of the method used in delivering the fetus (Fig. 5).

DISCUSSION

A number of studies have suggested that the delayed aggregation rate of human cord fibrin17,19 reflects production of a fetal fibrinogen qualitatively distinct from that in adult plasma. On the other hand, studies showing that cord
plasma specimens collected in the presence of a proteolytic inhibitor do not display a prolonged thrombin time,\(^1\)\(^8\)\(^9\) have suggested instead that proteolytic modification of fibrinogen in cord plasma could account for the observations. The present study has focused on these differing interpretations. First, like Guillin and Ménaché\(^1\)\(^2\), we have demonstrated that differences in aggregation rate between adult and cord fibrin are a function of the solvent conditions employed, particularly the ionic strength (Figs. 1, 2, Table 1). This finding therefore suggests that solvent dependency might have been an important factor when minimal or no differences were observed.\(^1\)\(^3\)\(^8\)\(^9\)\(^3\) Second, we have examined the possibility that the delay in aggregation displayed by cord fibrin might be due to postsynthetic proteolytic modification. Although coagulable fibrinogen catabolites (e.g., fraction 1-5) are present in both adult and cord plasma, it is clear from our experiments that their presence cannot account for the observed alterations in aggregation behavior displayed by cord fibrin (namely, prolonged clotting time and lower maximum absorbance, Figs. 1–3). This conclusion is consistent with the observations of Mills and Karpatkin\(^1\)\(^7\) on the clotting times of cord fibrinogen fractions.

Structural comparisons of cord and adult fibrinogen have thus far failed to disclose features that might account for the functional differences. Studies showing no structural differences have included comparison of the size of unmodified fibrinogen\(^5\) and its subunit chains (ref. 10 and this study), the electrophoretic behavior of unmodified fibrinogen\(^7\) or its S-carboxymethyl\(^1\) or S-sulfo (ref. 35 and this study) chains under acid and alkaline conditions, the immunochrometric behavior of unmodified fibrinogen and plasmic digests of fibrinogen\(^5\)\(^1\)\(^3\)\(^5\) or its S-sulfo derivatives,\(^3\)\(^5\) the amino acid content,\(^7\) the hexose content,\(^8\)\(^1\)\(^0\) and the peptide maps of tryptic hydrolysates of S-carboxymethyl derivatives.\(^1\)\(^3\)

Certain structural differences have been found, but their relationship to the functional characteristics of cord fibrinogen remains in question. Distinct tryptic peptide maps,\(^6\) increased anionic binding on DEAE-cellulose chromatography,\(^7\) and an increased phosphorus content\(^8\) of cord fibrinogen have been reported. Dephosphorylation of cord fibrinogen does not, however, correct its prolonged thrombin time.\(^9\) In this regard, since nascent adult fibrinogen has been shown to have a relatively higher phosphorus content,\(^3\) the high phosphorus content of cord fibrinogen may reflect immaturity of the dephosphorylating capacity of the fetus.

Cord fibrin clots are more transparent\(^2\)\(^1\)\(^2\) (Fig. 3) and have lower compressibility\(^2\) than those of the adult, an observation implying different structures. Since differences revealed by turbidimetry of adult and cord fibrin from fraction 1-2 persist in fibrin from fraction 1-5 (i.e., lower maximum absorbance), it appears that the COOH-terminal region of the Aα chain does not contain the structure(s) accounting for the unique behavior of cord fibrinogen. Although it is possible that catabolic processes other than those involving proteolytic attack on Aα chains may account for the differences between adult and cord fibrinogen, we favor the view that they are due to the expression of a “fetal” fibrinogen gene(s).
REFERENCES

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Evaluation of the role of in vivo proteolysis (fibrinogenolysis) in prolonging the thrombin time of human umbilical cord fibrinogen

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