Defective α-Polymerization in the Conversion of Fibrinogen Baltimore to Fibrin

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ABSTRACT The subunit structure of fibrinogen Baltimore and fibrin formed from this inherited dysfibrinogenemia was analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The molecular weights of the α-, β- and γ-chains of fibrinogen Baltimore were found to be identical to those of normal fibrinogen. Noncross-linked fibrin formed from both purified fibrinogen Baltimore as well as normal fibrinogen contained two α-monomers (α_1 and α_2). α_2 was presumed to be α-monomer from which fibrinopeptide A had been released. The evolution of α_2 during clotting of fibrinogen Baltimore was delayed and appeared to be quantitatively reduced when compared to normal. Cross-linked fibrin formed from fibrinogen Baltimore possessed an abnormal subunit structure. α-polymers were not generated in thrombin-induced, factor XIII-rich clots of fibrinogen Baltimore under conditions of pH and calcium concentration suitable for complete α-polymerization in normal fibrin. If clotting was carried out with calcium concentrations twice that required for normal clots or at pH 6.4, fibrin from fibrinogen Baltimore was completely cross-linked. These structural analyses of fibrin formed from fibrinogen Baltimore substantiate earlier findings that indicate a defect in the α-chain of this dysfibrinogenemia.

INTRODUCTION

Fibrinogen Baltimore is an inherited dysfibrinogenemia which is different from normal fibrinogen in its relative anodal migration on immunoelectrophoresis, delayed clotting by thrombin which is partially correctable by the addition of calcium or acidification to pH 6.4, and delayed release of fibrinopeptide A (1, 2).

Studies (3, 4) of human fibrinogen and fibrin utilizing polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS)[1] have revealed that normal fibrinogen consists of α-, β- and γ-polypeptide subunits with mol wt of approximately 70,000, 60,000, and 50,000, respectively. Fibrin formed in the absence of activated factor XIII (fibrin-stabilizing factor) or calcium is noncross-linked and contains α-, β-, and γ-monomers with molecular weights similar to those in fibrinogen, whereas fibrin formed in the presence of activated factor XIII and calcium (cross-linked fibrin) contains α-polymers (mol wt greater than 400,000), γ-dimers (mol wt approximately 105,000), and β-monomers.

We have employed SDS-polyacrylamide gel electrophoresis to study the subunit structure of fibrinogen Baltimore and the fibrin formed from this fibrinogen and find that fibrinogen Baltimore exhibits an abnormal α-chain pattern and defective α-polymerization when converted to fibrin.

METHODS

Venous blood from normal donors and a patient with fibrinogen Baltimore was collected in 1/10 vol of 3.8% sodium citrate and centrifuged at 1,900 *g* for 30 min to prepare plasma.

Factor XIII-rich fibrinogen containing trace amounts of plasminogen was purified from 50 to 100-ml portions of plasma by the method of Kazal, Amsel, Miller, and Tocantins (5). Plasminogen-free, factor XIII-rich fibrinogen was prepared by the method of Walker and Catlin (6). Purified fibrinogen was dissolved in 0.05 M sodium citrate, pH 7.4, and stored in lyophilized aliquots at −20°C. Clottability of purified fibrinogen, as determined by the method of Laki (7), was 95–97% for normal fibrinogen and 80–85% for fibrinogen Baltimore. Fibrinogen concentrations in plasma and purified preparations were determined by the methods of Clauss (8) and Ratnoff and Menzie (9).

Studies were carried out at pH 7.4 or 6.4. To control pH conditions all reagents were adjusted immediately before use to the appropriate pH by titration with 6 M hydrochloric acid or 10 M sodium hydroxide or by preparation

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[1] *Abbreviation used in this paper:* SDS, sodium dodecyl sulfate.

in barbital buffer (0.01 M sodium diethylbarbiturate, 0.015 M barbituric acid, 0.13 M sodium chloride) which had been adjusted to the appropriate pH with hydrochloric acid or sodium hydroxide.

Bovine thrombin (topical thrombin; Parke, Davis, & Co., Detroit, Mich.) used in determining fibrinogen concentrations and for preparing fibrin clots was dissolved at the desired concentration and pH in the appropriate barbital buffer. Highy purified bovine thrombin, kindly provided by Dr. A. R. Thompson, Harborview Medical Center, Seattle, Wash., was used in several studies to validate the effectiveness of crude thrombin. Such studies gave results identical to those in which crude thrombin was used; thus all results reported employed crude thrombin.

Factor XIII was purified from pooled human plasma by combining the methods of Lowey, Dunathan, Kriel, and Wolfinger (10), Mosesson and Umfleet (11), and Kazama and Langdell (12). This material was approximately 12,500-fold purified compared to original plasma and was stored in lyophilized aliquots at −20°C until used. For use, purified factor XIII was reconstituted in water in a concentration approximately 20 times that in the original plasma.

Fibrin was prepared from purified fibrinogen as follows: In 13 × 75 mm siliconized glass test tubes, 0.128 ml of fibrinogen (reconstituted at a final concentration of 2 mg/ml) was carefully adjusted to the desired pH with sodium hydroxide or hydrochloric acid, mixed with 0.064 ml of 0.02 M EDTA to prevent cross-linking or 0.064 ml of calcium chloride (final concentrations as indicated) to permit crosslinking, and clotted by the addition of thrombin (0.064 ml) at final concentrations ranging from 1.25 to 10 U/ml as indicated. To assess the effect of additional factor XIII on the subunit structure of clots formed from purified fibrinogen (already factor XIII-rich) in the presence of calcium, 0.016 ml of purified factor XIII, reconstituted for use as described above, was added to some samples before the addition of thrombin. Clots were allowed to form at 37°C for periods ranging from 5 min to 24 h after which they were washed in 0.15 M sodium chloride and frozen at −20°C until analyzed.

The subunit structure of purified fibrinogen and fibrin clots were analyzed by polyacrylamide gel electrophoresis in SDS by the method of Weber and Osborn (13) as modified by Schwartz, Pizzo, Hill, and McKee (4). Briefly, purified fibrinogen or clots prepared as above were incubated at a protein concentration of approximately 1 mg/ml for 12 h at 37°C in a 0.04 M sodium phosphate buffer, pH 7.1, which contained 9 M urea, 3% recrystallized SDS, and 3% β-mercaptoethanol. Electrophoresis was carried out for 4 h in 7.5% polyacrylamide gels with 15–40 μg of protein per gel. Gels were stained with Coomassie Blue and destained in a Quick Gel Destainer (Canalco, Inc., Rockville, Md.).

RESULTS

Clotting of fibrinogen Baltimore. The clotting characteristics of purified fibrinogen Baltimore were similar to those for plasma from the patient with this inherited dysfibrinogenemia. Results (Table I) indicated that the fibrinogen concentration of purified specimens of fibrinogen Baltimore was measured as exceedingly low when a test based on rate of clotting (Clauss method [8]) was used. However, when allowed to clot for 10 min in the presence of 10 U of thrombin per ml, fibrinogen Baltimore yielded a normal quantity of fibrin (Ratnoff and Menzie method [9]).

TABLE I

Fibrinogen Determinations on Plasma and Purified Fibrinogen from a Normal Control and a Patient with Fibrinogen Baltimore

Sample	Method	
	Clauss (8)	Ratnoff and Menzie (9)
	mg/ml	
Normal plasma	4.40	3.94
Patient's plasma	0.25	1.50
Purified normal fibrinogen	3.10	3.00
Purified fibrinogen Baltimore	0.10	2.63

Subunit structure of fibrinogen Baltimore. Based on migration in SDS-polyacrylamide gels, the α-, β-, and γ-subunits of fibrinogen Baltimore appeared to have molecular weights indistinguishable from those of normal fibrinogen (Fig. 1).

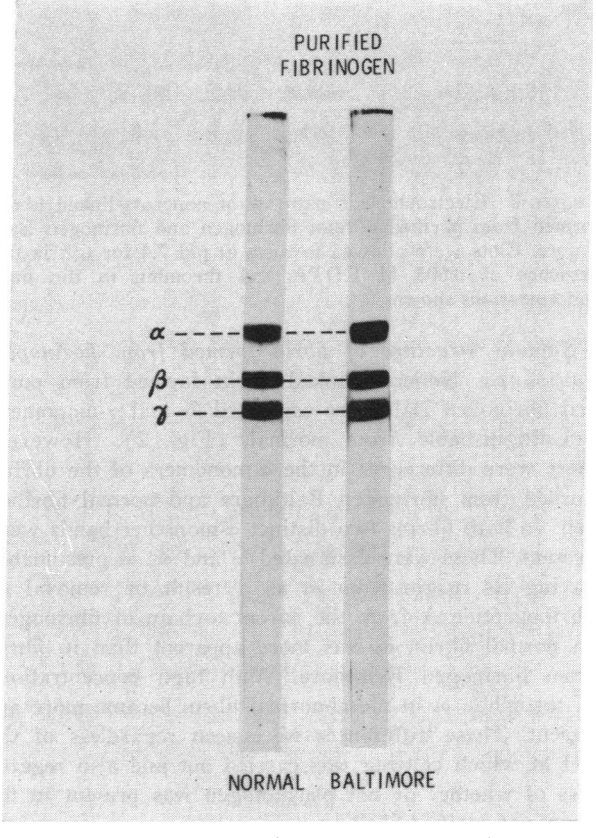

FIGURE 1 SDS-polyacrylamide gel electrophoretic patterns of purified normal fibrinogen and fibrinogen Baltimore. Lyophilized fibrinogen was reduced with β-mercaptoethanol in SDS and urea and electrophoresed on 7.5% polyacrylamide gels in SDS.

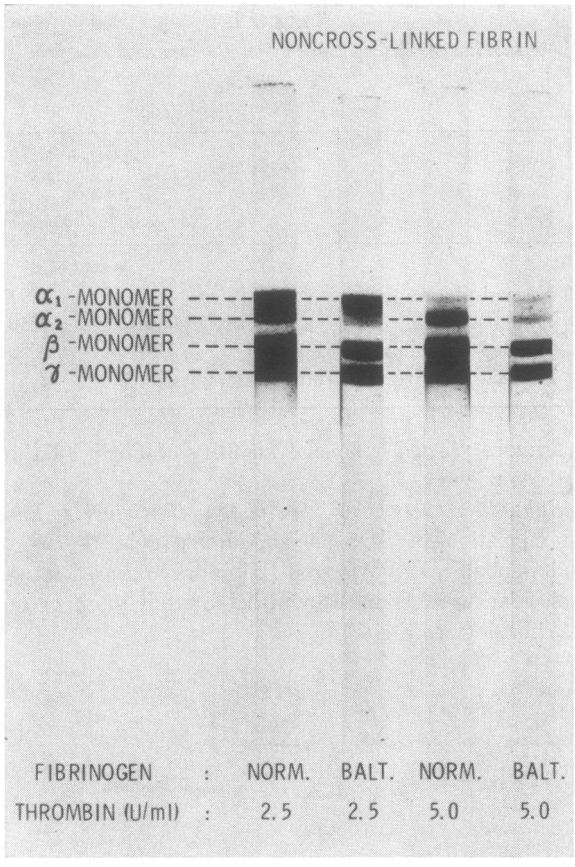

FIGURE 2 Electrophoretic patterns of noncross-linked fibrin formed from purified normal fibrinogen and fibrinogen Baltimore. Clots were allowed to form at pH 7.4 for 6 h in the presence of 0.005 M EDTA and thrombin in the final concentrations shown.

Subunit structure of fibrin formed from fibrinogen Baltimore. Noncross-linked fibrin formed from purified fibrinogen Baltimore contained β- and γ-monomers indistinguishable from normal (Fig. 2). However, there were differences in the α-monomers of the fibrins formed from fibrinogen Baltimore and normal fibrinogen. In both fibrins two distinct α-monomer bands were present. These were designated α_1 and α_2, α_2 presumably having its origin from α_1 as a result of removal of fibrinopeptide A from the parent α-chain of fibrinogen. In normal fibrin, α_2 was more apparent than in fibrin from fibrinogen Baltimore. With high concentrations of thrombin, α_2 in the abnormal fibrin became more apparent. These differences were seen regardless of the pH at which clotting was carried out and also regardless of whether or not plasminogen was present in the source of purified fibrinogen.

The subunit structures of cross-linked fibrin formed from normal fibrinogen and from fibrinogen Baltimore were markedly different (Fig. 3). Normal fibrin was fully cross-linked (i.e., only α-polymers, γ-dimers, and β-monomers were present) when clots were formed for 15 min at pH 7.4 in the presence of 0.00625 M calcium chloride and a final thrombin concentration of 1.25 U/ml. Fibrin from fibrinogen Baltimore prepared under the same conditions, except that samples were allowed to clot for 6 h, had no detectable α-polymers. Furthermore, α-monomers were still present in the abnormal fibrin. By increasing the concentration of calcium to 0.0125 M it was possible to fully polymerize the α-subunits of fibrinogen Baltimore. However, increasing the concentration of thrombin in the clotting mixtures only resulted in a diminution in the quantity of α-monomers with no enhancement of α-polymerization. This presumably occurred as a result of progressive degradation of α-chain in the presence of excess thrombin. One must assume that the peptides resulting from this degradation were not capable of polymerizing and were sufficiently small enough to escape from the gels during electrophoresis. The presence or absence of plasminogen did not affect the α-monomer pattern of cross-linked fibrin.

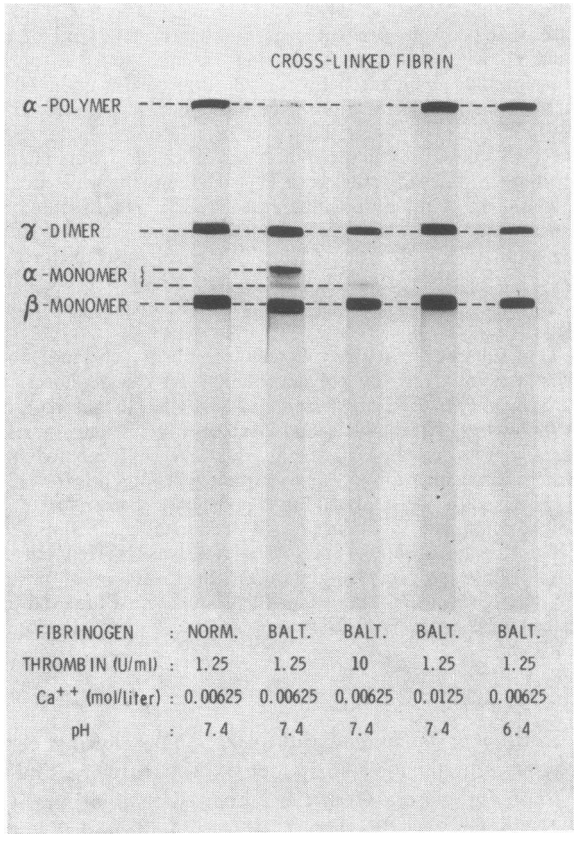

FIGURE 3 Electrophoretic patterns of cross-linked fibrin formed from purified normal fibrinogen and fibrinogen Baltimore. Normal fibrin was analyzed 15 min after the addition of thrombin and fibrin from fibrinogen Baltimore after 6 h. Environmental conditions of pH, calcium concentration, and thrombin concentration should be noted.

When clotting was carried out at pH 6.4 the defect in α-polymerization that occurred at pH 7.4 was no longer apparent (Fig. 3).

Increasing the concentration of factor XIII by the addition of purified factor XIII to clotting mixtures that contained factor XIII-rich fibrinogen Baltimore failed to correct the defect in α-polymerization. It should be noted that γ-dimerization always appeared to be normal in the clotting of fibrinogen Baltimore.

DISCUSSION

After its exposure to thrombin, fibrinogen is converted to a stable fibrin clot in a step-wise manner. Three distinct reactions occur. These include first, fibrinopeptide release, then monomer aggregation, and finally polymerization or cross-linking. Fibrinopeptide release results from the cleavage by thrombin of arginyl-glycine bonds at the NH_2-terminal end of the α- and β-chains of the fibrinogen molecule. The small peptide fragments removed during this reaction are designated fibrinopeptides A and B. After the release of these peptides fibrin monomers are formed which then aggregate into a gel. In the presence of calcium and activated factor XIII, covalent linking of α-chains into polymers and γ-chains into dimers will occur.

Abnormalities of clotting involving any one or more of these steps have been described. Several of the inherited dysfibrinogenemias exhibit defective fibrinopeptide release and/or abnormal monomer aggregation (14), and faulty clot stabilization exists in patients with inherited or acquired deficiences of factor XIII activity (15).

Beck, Charache, and Jackson and Beck, Shainoff, Vogel, and Jackson (1, 2) have shown that the release of fibrinopeptide A from fibrinogen Baltimore is delayed. They also present evidence that monomer aggregation is defective when fibrinogen Baltimore is clotted, but that clots formed this fibrinogen are insoluble in urea or monochloroacetic acid, indicating that cross-linking does occur at least to an extent that prevents dissolution of clots in these solvents.

The present studies in which SDS-polyacrylamide gel electrophoresis was used to analyze the subunit components of fibrinogen Baltimore and its fibrin reveal that while the molecular weights of α-, β-, and γ-chains of the abnormal fibrinogen are normal, both noncross-linked and cross-linked clots formed from fibrinogen Baltimore are structurally abnormal. Furthermore, all abnormalities detected in these studies involve only the α-chain.

Noncross-linked clots from fibrinogen Baltimore contained an α-monomer pattern different from normal in that the smaller molecular weight α-monomer, designated α_2, was retarded in appearance and quantitatively reduced. If α_2-monomer represents α-chain from which fibrinopeptide A has been released, our observations substantiate those of Beck and associates regarding defective fibrinopeptide A release from fibrinogen Baltimore. It does not appear that α_2-monomer is residual α-chain which has been partially digested by plasmin, because we also found α_2-monomer in fibrin prepared from plasminogen-free fibrinogen.

Cross-linked fibrin derived from the abnormal fibrinogen did not possess α-polymers when clotting was carried out under conditions of pH and calcium concentration that permitted α-polymerization in normal fibrin. This defect in α-polymerization was corrected by altering the pH to 6.4 or by increasing the calcium concentration, but not by adding additional thrombin or factor XIII.

Lorand (16) has suggested that the cross-linking of fibrin by the action of factor XIII and calcium depends upon the unmasking of cross-linking sites on the fibrinogen molecule during fibrinopeptide release. Because fibrinopeptide A release is abnormal during the clotting of fibrinogen Baltimore, it might be argued that α-polymerization in the fibrin deriving from this fibrinogen is defective because an insufficient number of cross-linking sites are exposed during clotting. To further speculate, when calcium and/or hydrogen ion concentrations are increased in the clotting mixture, perhaps an abnormal negatively charged locus in the α-chain is neutralized allowing for the unmasking of additional cross-linking sites. Evidence from the present study which would not support these theories is that even after seevral hours of clotting under conditions that should have permitted a normal quantity of fibrinopeptide A release no α-polymers appeared. Furthermore, Boyer, Shainoff, and Ratnoff (17) have shown that calcium ions actually retard fibrinopeptide release. They found that calcium accelerates fibrin formation by potentiating the aggregation of fibrin monomers.

We suggest, therefore, that the functional defect in fibrinogen Baltimore is the result of an abnormality of α-chain aggregation such that α-polymers are not produced under physiologic conditions. Because noncross-linked fibrin appears to be more friable and more easily lysed by plasmin than polymerized fibrin (16), perhaps the hemostatic defect associated with fibrinogen Baltimore resides in its failure to produce a clot sufficiently cross-linked to resist physiologic fibrinolysis. These studies do not explain why patients with fibrinogen Baltimore may encounter the problem of recurrent thromboembolism (1).

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