Rapid Formation of Large Molecular Weight α-Polymers in Cross-Linked Fibrin Induced by High Factor XIII Concentrations

Role of Platelet Factor XIII

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Abstract

After fibrin polymerization, activated Factor XIII catalyzes the formation of intermolecular cross-links between γ-chain pairs and also among two or more α-chains to form polymers. In this report we characterize the size and heterogeneity of α-chain polymers, establish the role of high concentrations of Factor XIII in determining the extent and rate of α-polymer formation, and also provide evidence that the Factor XIII required can be provided by platelets. Fibrin prepared from purified fibrinogen or platelet-deficient plasma contained a series of cross-linked α-chain polymers with M_r from 140,000 to 770,000 with a mean M_r difference of 32,000 consistent with a staggered, overlapping addition of monomers to the growing α-polymer chain. In plasma containing no platelets, α-polymer formation was incomplete with residual α-monomer remaining, but higher platelet counts facilitated more rapid cross-linking into larger polymers. Purified Factor XIII was equally effective as platelets in facilitating cross-linking. We conclude that cross-linked α-polymer chains are heterogeneous in size reaching a molecular weight of several million and that high concentrations of Factor XIII as provided by platelets are required for maximum cross-linking.

Introduction

The thrombin-catalyzed cleavage of fibrinopeptides from fibrinogen results in the formation of fibrin monomer that then self-assembles into a polymer through noncovalent interactions (1). Activated Factor XIII stabilizes the fibrin gel through formation of intermolecular ε-(γ-glutamyl) lysine covalent cross-links (2). One set of cross-links forms rapidly between two γ-chains of adjacent fibrin monomers with reciprocal antiparallel bonding between residues lys 406 and gln 398 near the carboxy terminus of the chain (3). Cross-linking of α-chain differs in its slower rate and greater complexity, with each α-chain forming a cross-link with up to two other α-chains, resulting in the formation of large polymers (4–7). The donor lys residues are located near the carboxy terminus of the α-chain and the acceptor gln residues, in the middle region (8–11), but the sequence and precise location of bonds formed during polymer formation have not been identified. The structure of the α-polymer chain is further complicated by cross-linking to it of other plasma proteins including α_2-plasmin inhibitor (12), fibronectin (13), and von Willebrand factor (vWf)[1] (14).

Plasma Factor XIII is an inactive enzyme precursor that circulates as a tetramer of two pairs of nonidentical a and b chains (15, 16). Activation of the zymogen is initiated by thrombin cleavage of a peptide from the two a chains that then dissociate from the b chains in the presence of calcium ions to form the active enzyme (15–18). Factor XIII is also found in the soluble fraction of platelets that normally contain ~ 50% of total blood Factor XIII activity (19–22). Plasma and platelet activated Factor XIII have the same enzymatic properties, but the platelet proenzyme differs from the plasma form in that it is composed of only a chain dimers (without b chains) (15, 16).

The contribution of α-chain polymer structure and size to fibrin strength and plasmin resistance is well established (23–25), as is the large potential size of α-polymers, based upon their nonentry into 5 or 7.5% polyacrylamide gels (4, 5). However, little is known of their polymeric structure or of the approximate upper limit of size of cross-linked α-chains in fibrin clots. In the present work, we have evaluated the size and heterogeneity of α-polymers in cross-linked fibrin prepared in vitro from fibrinogen and from plasma. A series of polymers is identified, including those of relative molecular weight equivalent to and exceeding those of plasma vWf multimers. The rate and extent of α-chain polymerization are found to be significantly influenced by Factor XIII concentrations above those found in normal plasma, whether provided by platelets or by additional purified Factor XIII. Since such high concentrations of Factor XIII would be available in vivo in regions of high platelet accumulation, formation of these polymers may significantly influence physiologic hemostasis and pathologic thromboembolic phenomena.

Methods

Protein preparation. Human fibrinogen (grade L) was obtained from Helena Laboratories (Beaumont, TX), was 93% clottable, and contained 2% fibronectin as determined by sodium dodecyl sulfate (SDS)–7% polyacrylamide gel electrophoresis of reduced protein and densitometry of protein-stained gels (Quickscan Jr.; Helena Laboratories). Fibrinogen concentration was determined by measurement of optical density at 280 nm using an extinction coefficient of 15.1 (26). Bovine thrombin (71 NIH U/mg) was obtained from Calbiochem-Behring Corp. (LaJolla, CA). Purified Factor XIII concentrate (fibrogammin), prepared from human placenta, was kindly provided by Behringwerke/Hoechst-Roussel (Somerville, NJ). Purified plasma Factor XIII was kindly provided by Dr. J. K. Smith, Plasma Fractionation Laboratory, Oxford, UK. Factor XIII was assayed by dansyl

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1. *Abbreviations used in this paper:* PDP, platelet-deficient plasma; vWf, von Willebrand factor.

cadaverine incorporation into casein (27) and expressed in relation to pooled normal plasma defined as 100%. Purified fibronectin was prepared from pooled normal plasma by gelatin Sepharose chromatography (28) and its concentration determined by measurement of optical density at 280 nm using an extinction coefficient of 12.8 (29). Fibrinogen and Factor XIII were depleted of fibronectin by gelatin Sepharose chromatography and showed no fibronectin band on overloaded reduced SDS–7% polyacrylamide gels. Cross-linked fibrin polymers were prepared by incubation of purified fibrinogen with low concentrations of thrombin as described by Connaghan et al. (30), and gluteraldehyde–cross-linked IgM polymers were prepared as described by Martin and colleagues (31). Bovine serum albumin and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO). Cross-linked fibrin digests containing fragments DD and DY were prepared as described previously (32). Goat antihuman fibrinogen and peroxidase-conjugated IgG fraction rabbit anti-goat IgG were obtained from Cappel Laboratories (Cochranville, PA).

Radiolabeling. Fibrinogen was labeled with the iodogen technique (33) to a specific activity of 0.5 μCi/mol using 0.02 mg of iodogen and 100 μCi ^{125}I/ml of fibrinogen (20 mg/ml), and unbound ^{125}I was separated by gel filtration chromatography on Sephadex G25 (Pharmacia Fine Chemicals, Piscataway, NJ). Labeled fibrinogen was 93% clottable and showed the same electrophoretic mobility on SDS–7% polyacrylamide gels after disulfide bond reduction.

Blood collection and processing. After obtaining informed consent, blood was obtained from normal donors and collected into sodium citrate (0.4% final concentration). To obtain platelet-rich plasma (PRP), blood was centrifuged at 160 *g* for 8 min at 25°C. Platelet-deficient plasma (PDP), which contained between 30,000 and 60,000 platelets/μl, was obtained by centrifugation of blood at 2,300 *g* for 15 min at 25°C. To further deplete plasma of platelets, the PDP was centrifuged at 12,000 *g* for 20 min at 25°C. Hemocytometer platelet counts were performed using phase contrast microscopy.

Clot preparation. Plasma containing 0.2 M epsilon aminocaproic acid and 1,000 kallikrein inhibitory units/ml of aprotinin (Mobay Chemical Corp., Pittsburgh, PA) was clotted by the addition of calcium chloride (50 mM final concentration) and thrombin (1 U/ml final concentration). Clots were allowed to incubate at 37°C for desired intervals, and the fibrin was separated from serum by pressing against the test tube wall with a glass rod and then rinsed three times with 0.15 M sodium chloride, 0.05 M Tris hydrochloric acid buffer, pH 7.6, containing 0.2 M epsilon aminocaproic acid and 1,000 kallikrein inhibitory units/ml aprotinin.

Electrophoresis. Electrophoresis in SDS–7% polyacrylamide gels was performed as described by Weber and Osborn (34). Polyacrylamide gradient gels of 2.2–6.5% were prepared, as described (35), using a gradient former (Model 570; Instrumentation Specialties Co., Lincoln, NE) with a 2% stacking gel utilizing a sulfate–borate discontinuous buffer system (36). Acrylamide and bisacrylamide were obtained from Bio-Rad Laboratories (Richmond, CA), and SDS for all purposes was obtained from Sigma Chemical Co. and recrystallized from ethanol for use. SDS–2% agarose gels were made using agarose (Seakem ME agarose) from FMC Corp. (Rockland, ME), as described (30). Protein staining was with Coomassie Brilliant Blue (Fisher Scientific Co., Allied Corp., Pittsburgh, PA), and autoradiograms were prepared from dried gels using Kodak X-omat (XAR-2) film (Eastman Kodak Co., Rochester, NY) with exposure times of 16–120 h. Fibrin was dissolved for electrophoresis with disulfide bond reduction by incubating in the SDS containing diluent appropriate for the gel system used at 60°C for 2–16 h. Western blotting was performed by a modification (37) of the method described by Towbin et al. (38). Multimeric analysis of plasma vWf was performed in 2% agarose gels using a modification (30) of the method described by Hoyer and Shainoff (39).

Results

The polypeptide chain composition of cross-linked fibrin was characterized by SDS electrophoresis using both acrylamide and agarose systems. In 7% polyacrylamide (Fig. 1 *A*), the purified fibrinogen showed heterogeneous Aα-chains and single Bβ- and γ-chains. Cross-linked fibrin prepared from pooled normal plasma or purified fibrinogen showed the β-chain migrating slightly further than the Bβ-chain of fibrinogen and virtual complete cross-linking of γ-chains to form dimers. The monomeric Aα-chains of fibrinogen were replaced by α-polymer forms represented primarily by a single nonentering band at the top of the gel and by several faint individual bands migrating more slowly than the γγ-chain.

Electrophoresis in 2.2–6.5% polyacrylamide gradient gels (Fig. 1 *B*) separated the nonentering band seen on 7% gels into a discrete series of bands migrating more slowly than the γγ, all of which reacted with antifibrinogen antiserum after Western blotting. Of 21 distinct bands, there were 5 heavy doublet bands at M_r of 140,000–260,000, 5 faint bands with M_r between 280,000 and 360,000, and at least 11 prominent bands migrating with apparent M_r of 420,000–700,000. The mean M_r difference between individual bands was 32,000±12,000 (SD) with an M_r range of 20,000–60,000. Clots made with fibrinogen and Factor XIII, both of which had been depleted of fibronectin, had the same gel pattern (not shown).

Incubation of clots made from fibrinogen or PDP for > 3 h resulted in a blurred band pattern, and a greater proportion of more slowly migrating protein appeared. This progression was most evident with SDS–2% agarose gels (Fig. 1 *C*). At least 6 individual faint bands could be identified between the γγ band and the 680,000-mol-wt marker at 16 h, and some protein migrated at least as slowly as cross-linked trimer of IgM (M_r of 2,750,000), which was the largest molecular weight marker used. The extent of α-chain cross-linking in fibrin prepared from plasma was influenced by the platelet count. Compared with fibrin clots of PDP with a platelet count between 30,000 and 60,000/μl (Fig. 1 *C*), fibrin prepared from PRP (platelet count 700,000/μl) showed larger α-polymer forms (Fig. 2). These encompassed the range of sizes of vWf multimers and also a portion that migrated more slowly than the largest normal plasma vWf multimers and some that even failed to enter the 2% agarose gel.

In fibrin prepared from plasma with low platelet counts, cross-linking of α-chains was incomplete, with residual α-monomer still present (Fig. 3). Radiolabeled fibrinogen was added to the clotting mixture so that fibrinogen-derived protein could be distinguished from other plasma proteins by autoradiography and to enable quantitation of polypeptide chains after gel slicing. The autoradiograms showed complete γ-dimerization in all clots and no changes in the β-chain due to cross-linking. However, residual α-monomer chains present in clots prepared in the absence of platelets progressively decreased with platelet counts of 200,000/μl and 420,000/μl and were very faint in clots with a platelet count of 900,000/μl. The amount of the largest α-polymers increased progressively at higher platelet counts in proportion to the decrease in α-monomer. Intermediate α-polymers, which entered the gel but migrated more slowly than the γγ-chain, were also less prominent in clots prepared with higher platelet counts of 420,000/μl and 900,000/μl. The pattern was identical in clots in which the platelets had been lysed by freezing and thawing before clot preparation (not shown). To quantitate the change in α-monomer, the gels were cut up and individual slices counted. The proportion of α-monomer was 43% in clots prepared with no platelets, and progressively less in fibrin prepared at higher

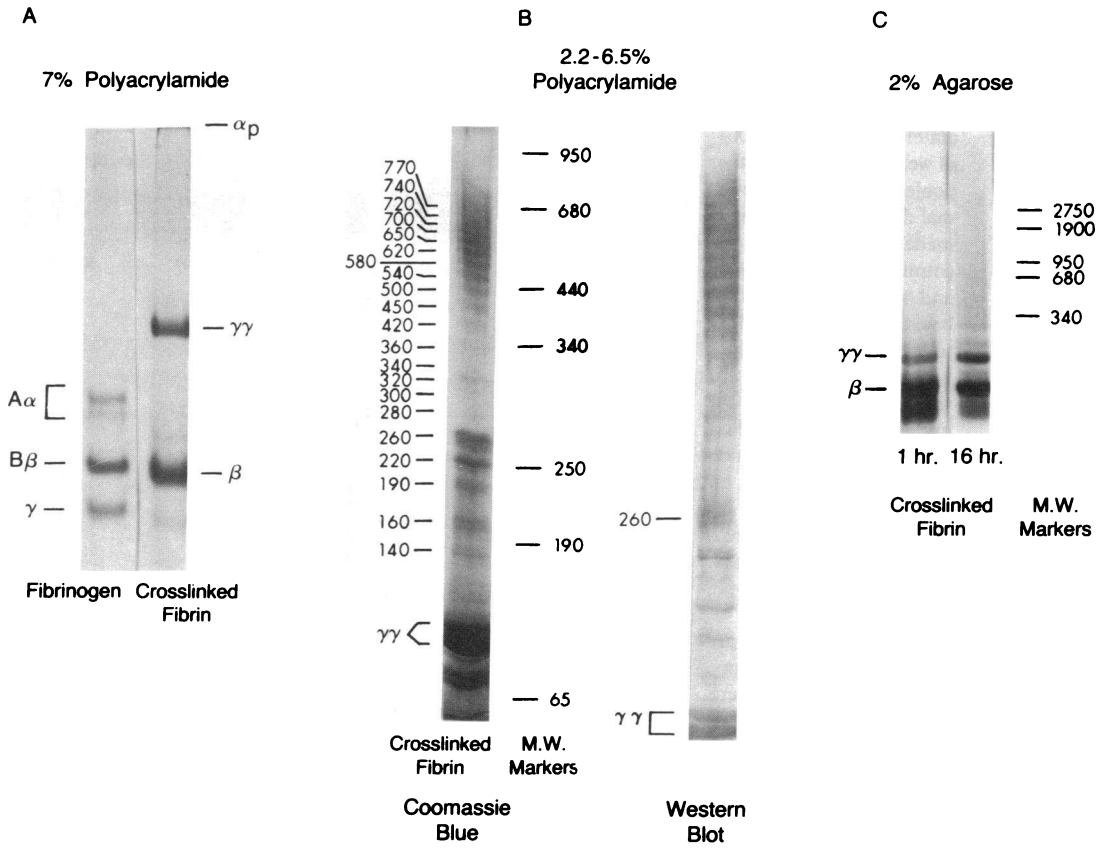

Figure 1. Electrophoresis of reduced cross-linked fibrin in different gel systems. (*A*) SDS–7% polyacrylamide gel of purified fibrinogen and a 1-h fibrin clot prepared from pooled normal plasma (10 μg each). α-polymer chains are represented by nonentering protein at the top of the gel and by several faint bands migrating more slowly than the γγ-chain. (*B*) Electrophoresis in an SDS–2.2–6.5% polyacrylamide gel of fibrin (60 μg) prepared by clotting pooled normal plasma for 1 h. The 2% stacking gel was lost in preparation. Electrophoresis proceeded until the γγ-chains were near the bottom of the gel and the β-chain had exited. The locations of unreduced molecular weight standards electrophoresed on the same gel are indicated and include bovine serum albumin (65,000 mol wt), cross-linked fibrin fragment DD (190,000 mol wt), cross-linked fibrin fragment DY (250,000 mol wt), fibrinogen (340,000 mol wt), fibronectin (440,000 mol wt), fibrin dimer (680,000 mol wt), and IgM (950,000 mol wt). The lane to the right shows a Western blot of the same sample using antifibrinogen antiserum. Because of differences in shrinkage during staining or blotting, the lane lengths are different. The locations of the γγ-bands and of the 260,000-mol-wt α-polymer band are indicated for reference. The MW markers refer to the Coomassie Blue–stained gel strip only. (*C*) SDS–2% agarose gel. Fibrin (10 μg) was prepared from pooled normal plasma clotted for 1 or 16 h. The unreduced molecular weight markers used were fibrinogen 340,000, fibrin dimer 680,000, IgM 950,000, IgM dimer 1,900,000, and IgM trimer 2,750,000.

platelet counts, reaching a minimum of 13% at 900,000/μl (Fig. 3 *B*).

The effect of platelet count on the rate as well as the extent of α-polymer formation is shown in Fig. 4, using SDS–2% agarose gels. A greater proportion of progressively larger α-polymer chains was seen in fibrin prepared from plasma at higher platelet counts. At platelet counts of 3,000/μl and 11,000/μl a very small quantity of slowly migrating α-polymer formed; on the other hand, at platelet counts of 113,000/ml and 500,000/μl there was a progressive increase in larger α-polymer and at 500,000/μl some failed to enter the gel. There was a clear trend toward higher molecular weight α-polymer forms in clots prepared for longer times, particularly those with platelet counts of 39,000/μl and 113,000/μl. At lower platelet counts the time dependance was less evident, whereas at a platelet count of 500,000/μl extensive polymerization was already present at 1 h. At all platelet counts, maximum cross-linking was present in clots incubated for 24 h with no further increase in apparent size or change in distribution of α-polymer sizes with longer incubations of up to 5 d.

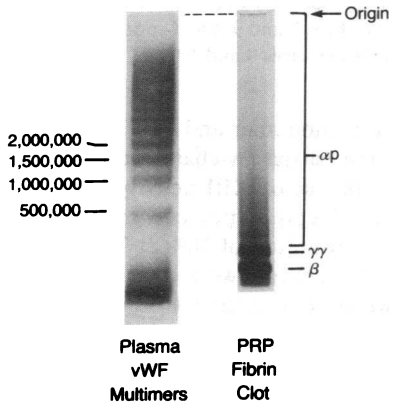

Figure 2. SDS–2% agarose electrophoresis of PRP fibrin clot and plasma vWf multimers. *Left,* autoradiogram showing vWf multimers in normal plasma after electrophoresis (unreduced) and overlay with ^{125}I anti-vWf IgG. Migration positions and approximate molecular weight of the smallest multimers are indicated. *Right,* autoradiograms of gel of reduced cross-linked fibrin prepared from normal PRP (platelet count 700,000/μl) to which ^{125}I radiolabeled fibrinogen was added and clotted for 3 h. Both gel strips are from the same gel and the location of the application wells is indicated as "Origin." Changes in size during separate processing account for the difference in gel length.

The distribution of radioactivity in gels of 8-h clots was determined after slicing and counting (Fig. 4 *B*). In this gel system α-monomer and lower molecular weight α-polymer forms migrating near the γγ-chain could not be clearly distinguished. However, there was a progressive shift in the distribution of counts toward those with slower mobilities in clots made with a higher platelet content. In fibrin prepared from plasma with a platelet count of 500,000/μl, 36% of the counts

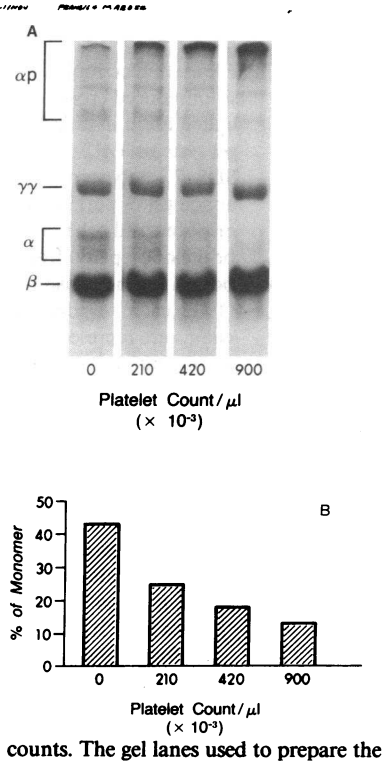

Figure 3. (*A*) Gel electrophoresis of fibrin prepared from plasma with different platelet counts. Plasma containing no identifiable platelets and PRP with a platelet count of 900,000/μl were prepared from the pooled blood of five normal donors; mixtures were made to adjust platelet counts between 210,000/μl and 420,000/μl. Radiolabeled fibrinogen was added to the plasma and clots were incubated for 3 h after the addition of thrombin and calcium chloride. Fibrin was dissolved with disulfide bond reduction, and electrophoresis was in SDS–7% acrylamide gels. Autoradiograms were exposed for 24 h. (*B*) Proportion of monomeric α chains in plasma clots prepared at varying platelet counts. The gel lanes used to prepare the autoradiograms in *A* were cut into slices and counted for radioactivity. The location of α-monomer was identified by protein staining. Total α-chain was taken as the sum of α-monomer and all counts migrating more slowly than the γγ-chain.

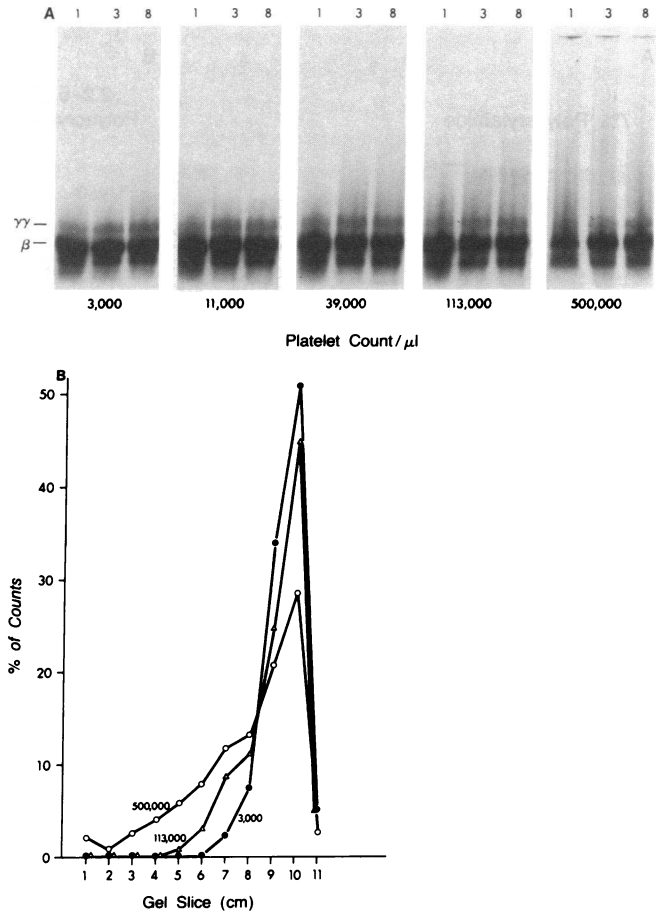

Figure 4. (*A*) SDS–2%-agarose electrophoresis of fibrin clots prepared with increasing platelet counts and for different incubation intervals. PRP and plasma with a platelet count of 3,000/μl were prepared from five normal donors and mixtures made to adjust the platelet counts as shown. After addition of radiolabeled fibrinogen, clots were prepared for 1, 3, and 8 h; dissolved; and aliquots subjected to electrophoresis. A total of 60,000 counts was applied to each lane, and autoradiography was for 20 h. (*B*) Distribution of radioactivity in polypeptide chains of fibrin prepared for 8-h plasma clots at varying platelet counts after SDS–2% agarose gel electrophoresis. 8-h plasma clots prepared from plasma with platelet counts of 3,000/μl, 113,000/μl, and 500,000/μl were electrophoresed as shown in *A*. After staining, drying, and autoradiography, the gel was sliced at intervals and the proportion of radioactivity in each slice calculated. The γγ-chains were located in slices 8 and 9, the α-monomer in slices 9 and 10, and β-chain also in slices 9 and 10.

migrated in gel slices 1–7, compared with 13% and 3% in clots made in the presence of 113,000/μl and 3,000/μl, respectively. Correspondingly, the counts in slices 9 and 10 decreased, reflecting loss of α-monomer.

Since platelet Factor XIII could account for the difference in cross-linking, clots were prepared from plasma containing no platelets, but supplemented with purified Factor XIII (Fig. 5). The clot prepared from plasma of a patient congenitally deficient in Factor XIII showed only partial γ-chain cross-linking on 7% polyacrylamide gels consistent with a low residual Factor XIII level (now shown). Similarly, on the SDS–2% agarose gel, the γ-dimer formation was incomplete, and there was no staining above the γγ-band indicating little formation of large α-polymers. Fibrin prepared at 26, 54, and 80% Factor XIII concentration showed increasing amounts of α-polymer bands that were slightly larger than the γγ-chain, but little of that migrated more slowly than the fibronectin subunit (M_r of 220,000). At Factor XIII concentrations between 127 and 235% the amount of more slowly migrating α-polymer increased, including some protein that did not enter the gel in the 235% sample. The nonentering α-polymer was increasingly prominent in fibrin prepared with Factor XIII concentrations of 346–708%. At the highest concentrations of 600 and 708%, most of the α-polymer was of such large size that it failed to enter the 2% gel. The addition of leupeptin (100 μg/ml final concentration) during clot preparation to inhibit platelet calpain did not affect the extent of α-chain polymerization as shown in Fig. 3 nor the size of polymers (Fig. 4).

Samples of reduced clots were also electrophoresed on SDS–7% acrylamide gels, and the proportions of α-chain migrating as monomer and polymer were determined after counting of gel slices (Fig. 6). In Factor XIII–deficient plasma, 78% of α-chain migrated as monomer and 22% as polymer, findings consistent with the partial γγ-chain formation and indicative of a low level of Factor XIII activity. At higher Factor XIII concentrations, the proportion of α-monomer decreased progressively to a minimum of 10% at 708% Factor XIII. The polymeric α-chain forms were divided into two fractions, the largest of which were in the 1st 1-cm slice of gel, and with intermediate-sized polymers migrating further, but more slowly than the γγ-chain marker. The largest α-polymer forms increased progressively to reach 67% of total α-chains at 708% Factor XIII. The intermediate α-polymers reached a peak of 41% at 80% Factor XIII and then declined at higher Factor XIII concentrations. Similar results were obtained by adding purified plasma Factor XIII. At 200% Factor XIII, α-monomers represented 13% of the total; large polymers, 60%;

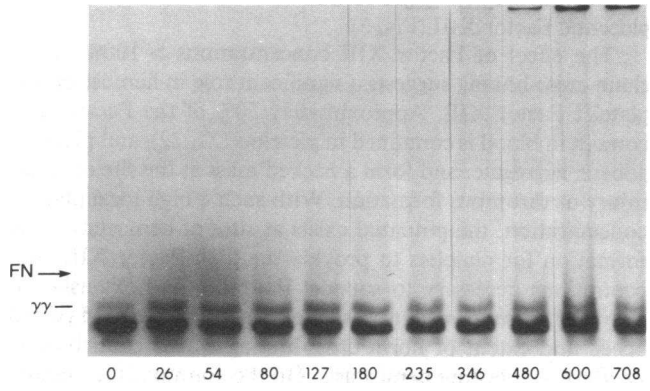

Figure 5. SDS–2% agarose electrophoresis of 3-h plasma clots prepared with increasing Factor XIII concentrations. Pooled plasma from five normal donors was centrifuged to prepare plasma containing no identifiable platelets, and the Factor XIII activity in this standard sample was taken to be 100%. Plasma containing 0% Factor XIII was obtained from a patient with a severe congenital deficiency of Factor XIII. Plasmas with Factor XIII concentrations of 26, 54, and 80% were obtained by mixing the platelet-free normal plasma and the Factor XIII–deficient plasma. Plasma containing Factor XIII concentrations above 100% was prepared by adding a Factor XIII concentrate to the standard plasma. The Factor XIII concentrations shown were determined by direct assay of plasma. Approximately 60,000 counts were added to each lane and the autoradiogram was exposed for 24 h. FN indicates the migration position of the fibronectin subunit (M_r of 220,000) located on the protein-stained gel but not seen on the autoradiogram.

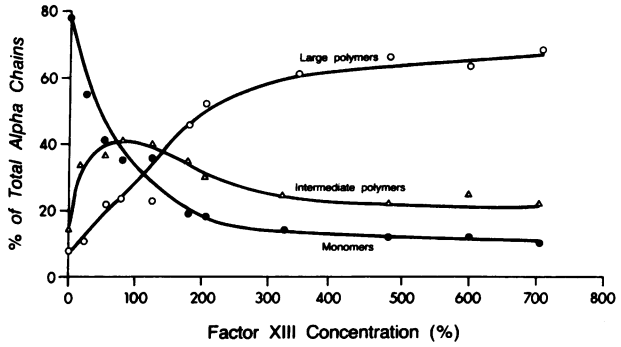

Figure 6. Proportion of monomeric and polymeric α-chain forms in fibrin prepared at increasing Factor XIII concentrations. Fibrin was prepared from plasma with varying concentrations of Factor XIII and electrophoresed in SDS–7% polyacrylamide gels. After staining, drying, and autoradiography the proportion of counts migrating in different positions was determined. The α-monomer was taken to represent counts between the γγ- and β-chains. The largest α-polymers were those contained in the 1st 1 cm, and intermediate sized α-polymer forms were those migrating from 1 cm to the γγ-chain position. Factor XIII concentrations were determined by assay. Factor XIII concentrations between 0 and 100% were adjusted by mixing Factor XIII–deficient plasma with pooled normal plasma containing no platelets, whereas Factor XIII concentrations above 100% were achieved by adding purified placental Factor XIII. Proportions of α-monomer, intermediate polymers, and large polymers are expressed in comparison to the total α-chain content in each sample.

and intermediate forms, 27%; whereas at a Factor XIII concentration of 700%, the percentages were 10, 70, and 20%, respectively.

Discussion

Our findings demonstrate that α-polymer chains of cross-linked fibrin are heterogeneous and may be exceedingly large in size, of equal or greater molecular weight than vWf multimers, and also show that the rate and extent of α-polymer formation are critically dependent on Factor XIII concentration, especially that derived from platelets. Thus, fibrin prepared from pooled normal plasma with 100% Factor XIII concentration showed residual α-monomer (Fig. 3) and α-polymer forms of smaller (Fig 4 and 5) size, whereas progressive cross-linking to larger polymeric forms required either added purified Factor XIII or higher platelet counts.

The slow formation of α-chain polymers in cross-linked fibrin was first demonstrated by McKee and colleagues (4) who also showed that γ-chains are rapidly cross-linked to dimers. The large size of α-polymers has been inferred from their failure to enter 5 or 7.5% polyacrylamide gels and explained by the capacity of Factor XIII to cross-link each α-chain with up to two others (4–7), thereby creating the potential for unlimited size. The present study extends these findings, defining the size and heterogeneity of α-polymer chains through the use of gel electrophoretic systems of high porosity and resolution.

The heterogeneity of α-polymers is demonstrated in clots of limited α-polymerization, obtained from platelet-deficient plasma or from purified fibrinogen. Electrophoresis of the reduced fibrin in 2.2–6.5% polyacrylamide gels shows 21 α-polymer forms with M_r of 140,000–770,000 (Fig. 1 *B*). The large size of the polymers precludes their derivation from β- or γ-chains, as does the inverse correlation of α-monomer disappearance with α-polymer formation (Figs. 3 and 6). With longer incubation intervals the bands were less distinct and larger α-polymers formed (Fig. 1 *C*), perhaps the result of progressive cross-linking of smaller α-polymers to larger forms that could not be individually resolved on the 2% agarose gel system. The exact molecular weight of these polymeric forms (Fig. 1 *B*) cannot be accurately established because of the paucity of appropriate molecular weight markers and because the cross-linking of both α-polymer chains and available markers may result in aberrant migration on SDS gels. However, the electrophoretic pattern suggests a regular polymeric series with an average M_r difference between successive components of 32,000. Considering a M_r of the α-chain of 66,000 (40), this pattern would initially appear inconsistent with a simple additive polymeric series. However, since the lysyl cross-link donor residues are located at the carboxy-terminal end of the chain and glutaminyl acceptors in the middle portion (8–11), the possibility of chain overlap during cross-linking exists, as has been suggested by previous models (8, 10). Therefore, the polymeric series demonstrated in Fig. 1 *B* may represent a half-overlap series of α-chains, with the apparent difference between successive polymers being about one-half of the molecular weight of α-chain monomers. According to this scheme, the band with migration corresponding to M_r of 140,000 (Fig. 1 *B*) would represent an α-chain trimer, and the dimeric form would migrate with an apparent M_r of ~ 99,000 so that it would be obscured by the γγ-bands. Up to 22 monomers would be required to reach the size of the 770,000 form and several fold more to produce the largest α-polymers of a size approximating and exceeding large vWf multimers (Fig. 2). Additional factors must influence the electrophoretic distribu-

tion since some α-polymers are more prominent, especially those of M_r of 140,000–220,000 and 420,000–770,000, whereas others are present in lower amount (Fig. 1 *B*).

An additional source of polymer size heterogeneity may be the variability in size of α-chains in plasma or purified fibrinogen preparations due to proteolytic degradation. Fibrinogen in freshly drawn blood contains ~ 11% degraded Aα-chains (41), the majority of which have lost an ~ 3,000-mol-wt fragment from the carboxy-terminal end. Cross-linking of a degraded α-chain would produce a polymer of M_r of 3,000 less than expected, but this should not affect significantly the size or electrophoretic mobility. However, degradation that cleaves the sites involved in cross-linking could limit the maximal size of α-polymers and also could explain the 10% residual uncross-linked fibrin monomer seen in fibrin clots made with high platelet counts (Fig. 3 *B*) or high Factor XIII concentration (Fig. 6).

Other plasma proteins, including α_2-plasmin inhibitor (12), fibronectin (13), and vWf (14), cross-link to fibrin α-chains and could contribute to structural heterogeneity. Hada and colleagues (14) found cross-linking of up to 60% of plasma vWf into fibrin formed after clotting of normal plasma for 3 h. This would yield a maximum molar ratio of vWf 200,000-mol-wt subunit to fibrin α-chain of 1:400, considering a plasma vWf concentration of 10 μg/ml (42) and plasma fibrinogen concentration of 7.3 μM (1). Although vWf incorporation may influence the kinetics of α-polymer formation (14), this low molar incorporation would be unlikely to substantially alter the electrophoretic pattern. The normal plasma concentration of α_2-plasmin inhibitor is 1 μM and a maximum of 30% cross-links to fibrin (12), resulting in a maximum molar ratio to α-chain of ~ 1:50. This binding would also have little influence on the electrophoretic results. Mosher and colleagues (13) have identified cross-linked fibronectin/α-chain hybrids by SDS polyacrylamide gel electrophoresis of cross-linked fibrin, but fibronectin did not affect the formation of large α-polymers noted in this study, since the same results were obtained with fibronectin-depleted fibrinogen. Thus, the effect of cross-linking of vWf, α_2-plasmin inhibitor, and fibronectin to α-chains would be to introduce additional heterogeneity to the α-polymers, but the low molar ratios preclude recognition of possible hybrid forms using our gel techniques.

Maximal α-chain cross-linking was facilitated by higher platelet counts or Factor XIII concentrations than are present in normal plasma (Figs. 3–6). In plasma containing no platelets, 43% of α-monomer remained uncross-linked in clots incubated for 3 h, but α-monomer decreased to 10% at a platelet count of 420,000/μl and virtually disappeared with 900,000 platelets/μl (Fig. 3 *B*). The larger α-polymers that formed at higher platelet counts (Fig. 4) did not require metabolically active or even intact platelets since the same results were obtained with platelets lysed by freezing and thawing. That this facilitation by platelets was due to their content of Factor XIII is supported by experiments using supplemental placental Factor XIII, which has the same structure as platelet Factor XIII (43). Addition of placental Factor XIII to plasma containing no platelets resulted in an identical progressive acceleration of α-polymer formation and increase in polymer size as was obtained with platelets (Fig. 5). The effect of platelets on cross-linking could be accounted for by their Factor XIII content considering that plasma with a platelet count of 300,000/μl had 200% Factor XIII concentration. Thus, α-polymer formation at a platelet count of 500,000/μl with a total Factor XIII concentration of 265% (Fig. 4 *A*) is comparable to that with a similar Factor XIII concentration provided by purified placental Factor XIII (Fig. 5).

The effect of Factor XIII concentrations > 100% on α-chain cross-linking suggests a significant role in hemostasis for platelet Factor XIII. Approximately 50% of the Factor XIII content in blood is contained in platelets (21, 22), and platelets adhere, aggregate, and form a packed mass at the site of vessel injury or thrombus formation. With such a high local platelet concentration, the potential exists at sites of hemostatic plug formation for platelets to provide the high Factor XIII concentrations necessary to support the rapid and extensive α-polymer formation demonstrated in this report. Maximal speed and extent of fibrin cross-linking would contribute to effective and lasting hemostasis. On the contrary, the absence of sufficient platelet numbers would produce defective α-chain polymerization, as observed by Rodeghiero and colleagues (44) in patients with acute leukemia, and possibly contribute to a bleeding diathesis.

The extent of α-chain polymer formation influences the physical properties of fibrin clots by increasing the degree of cross-linking resulting in greater rigidity and elasticity (45, 46), and greater resistance to plasmic degradation (23–25). However, these studies assessed cross-linking with gel systems of lower resolution did not define the α-polymers of the size demonstrated in this report, and the effect of Factor XIII concentrations > 100% was not considered. Therefore, the functional significance of progressive α-chain cross-linking to the very large polymers demonstrated here is not known. However, clinical observations indicate that newly formed thrombi are susceptible to pharmacologic lysis with plasminogen activators, whereas older thrombi become progressively resistant (47, 48). Possibly, the progressive cross-linking of α-chains to large molecular weight polymers dependent on high level Factor XIII concentration may be an important determinant of susceptibility of thrombi to lysis.

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References

1. Doolittle, R. F. 1984. Fibrinogen and fibrin. *Annu. Rev. Biochem.* 53:195–229.

2. Folk, J. E., and J. S. Finlayson. 1977. The (γ-glutamyl) lysine crosslink and the catalytic role of transglutaminase. *Adv. Protein Chem.* 31:1–133.

3. Chen, R., and R. F. Doolittle. 1971. γ-γ cross-linking sites in human and bovine fibrin. *Biochemistry.* 10:4486–4491.

4. McKee, P. A., P. Mattock, and R. L. Hill. 1970. Subunit structure of human fibrinogen, soluble fibrin, and cross-linked insoluble fibrin. *Proc. Natl. Acad. Sci. USA.* 66:738–744.

5. Schwartz, M. L., S. V. Pizzo, R. I. Hill, and P. A. McKee. The effect of fibrin-stabilizing factor on the subunit structure of human fibrin. *J. Clin. Invest.* 50:1506–1513.

6. Pisano, J. J., J. S. Finlayson, M. P. Peyton, Y. Nagai. 1971. ε-(γ-glutamyl) lysine in fibrin: lack of crosslink formation in factor XIII deficiency. *Proc. Natl. Acad. Sci. USA.* 68:770–772.

7. Ball, A. P., and P. A. McKee. 1977. Fibrin formation and dissolution in women receiving oral contraceptive drugs. *J. Lab. Clin. Med.* 89:751–763.

8. Doolittle, F. E., K. G. Cassman, B. A. Cottrell, and S. J. Friezner. 1977. Amino acid sequence studies on the α chain of human fibrinogen. Isolation and characterization of two linked α-chain cyanogen bromide fragments from fully cross-linked fibrin. *Biochemistry.* 16:1715–1719.

9. Fretto, L. J., E. W. Ferguson, H. M. Steinman, and P. A. McKee. 1978. Localization of the α-chain cross-link acceptor sites of human fibrin. *J. Biol. Chem.* 253:2184–2195.

10. Fretto, L. J., and P. A. McKee. 1978. Structure of α-polymer from *in vitro* and *in vivo* highly cross-linked human fibrin. *J. Biol. Chem.* 253:6614–6622.

11. Cottrell, B. A., D. D. Strong, K. W. K. Watt, and R. F. Doolittle. 1979. Amino acid sequence studies on the α-chain of human fibrinogen. Exact location of cross-linking acceptor sites. *Biochemistry.* 18:5405–5410.

12. Sakata, Y., and N. Aoki. 1980. Cross-linking of α_2-plasmin inhibitor to fibrin by fibrin-stabilizing factor. *J. Clin. Invest.* 65:290–297.

13. Mosher, D. 1975. Cross-link of cold-insoluble globulin by fibrin-stabilizing factor. *J. Biol. Chem.* 250:6614–6621.

14. Hada, M., M. Kaminski, P. Bockenstedt, and J. McDonagh. 1986. Covalent crosslinking of von Willebrand factor to fibrin. *Blood.* 68:95–101.

15. Schwartz, M. L., S. V. Pizzo, R. L. Hill, and P. A. McKee. 1973. Human factor XIII from plasma and platelet. Molecular weights, subunit structures, proteolytic activation, and cross-linking fibrinogen and fibrin. *J. Biol. Chem.* 248:1395–1407.

16. Chung, S. I., M. S. Lewis, and J. E. Folk. 1974. Relationships of the catalytic properties of human plasma and platelet transglutaminases (activated blood coagulation factor XIII) to their subunit structures. *J. Biol. Chem.* 249:940–950.

17. Curtis, C. G., K. L. Brown, R. B. Credo, R. A. Domanik, A. Gray, P. Sternberg, and L. Lorand. 1974. Calcium-dependent unmasking of active center cysteine during activated fibrin stbilizing factor. *Biochemistry.* 13:3774–3780.

18. Takagi, T., and R. F. Doolittle. 1974. Amino acid sequence studies on factor XIII and peptide released during its activation by thrombin. *Biochemistry.* 13:750–756.

19. Buluk, K. 1955. An unknown function of blood platelets. *Pol. Tyg. Lek.* 10:191–198.

20. Kiesselbach, T. H., and R. H. Wagner. 1966. Fibrin-stabilizing factor: a thrombin-labile platelet protein. *Am. J. Physiol.* 211:1472–1476.

21. McDonagh, J., R. P. McDonagh, J.-M. Delage, and R. H. Wagner. 1969. Factor XIII in human plasma and platelets. *J. Clin. Invest.* 48:940–945.

22. Lopaciuk, S., K. M. Lovette, J. McDonagh, H. Y. K. Chuang, and R. P. McDonagh. 1976. Subcellular distribution of fibrinogen and factor XIII in human blood platelets. *Thromb. Res.* 8:453–465.

23. Gormsen, J., A. P. Fletcher, N. Alkjaersig, and S. Sherry. 1967. Enzymic lysis of plasma clots: the influence of fibrin stabilization on lysis rates. *Arch. Biochem. Biophys.* 120:654–665.

24. R. P. McDonagh, Jr., J. McDonagh, and F. Duckert. 1971. The influence of fibrin crosslinking on the kinetics of urokinse-induced clot lysis. *Br. J. Haematol.* 21:323–332.

25. Gaffney, P. J., and A. N. Whitaker. 1979. Fibrin crosslinks and lysis rates. *Thromb. Res.* 14:85–94.

26. Marder, V. J., N. R. Shulman, and W. F. Carroll. 1969. High molecular weight derivatives of human fibrinogen produced by plasmin. I. Physicochemical and immunological characterization. *J. Biol. Chem.* 244:2111–2119.

27. Lorand, L., T. Urayama, J. W. C. deKiewiet, and H. L. Nossel. 1969. Diagnostic and genetic studies on fibrin-stabilizing factor with a new assay based on amine incorporation. *J. Clin. Invest.* 48:1054–1064.

28. Engvall, E., and E. Ruoslahti. 1977. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer.* 20:1–5.

29. Mosesson, M. W., and R. A. Umfleet. 1970. The cold-insoluble globulin of human plasma. I. Purification, primary characterization, and relationship to fibrinogen and other cold-insoluble fraction components. *J. Biol. Chem.* 245:5728–5736.

30. Connaghan, D. G., C. W. Francis, D. A. Lane, and V. J. Marder. 1985. Specific identification of fibrin polymers, fibrinogen degradation products, and crosslinked fibrin degradation products in plasma and serum with a new sensitive technique. *Blood.* 65:589–597.

31. Martin, S. E., V. J. Marder, C. W. Francis, L. S. Loftus, and G. H. Barlow. 1980. Enzymatic degradation of the factor VIII-von Willebrand protein: a unique tryptic fragment with ristocetin cofactor activity. *Blood.* 55:848–858.

32. Francis, C. W., V. J. Marder, and S. E. Martin. 1980. Plasmic degradation of crosslinked fibrin. I. Structural analysis of the particulate clot and identification of new macromolecular soluble complexes. *Blood.* 56:456–464.

33. Fraker, P. J., and J. C. Speck, Jr. 1978. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* 80:849–857.

34. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244:4406–4412.

35. Francis, C. W., V. J. Marder, and S. E. Martin. 1979. Detection of circulating crosslinked fibrin derivatives by a heat extraction-SDS gradient gel electrophoretic technique. *Blood.* 54:1282–1295.

36. Neville, D. M., Jr. 1971. Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. *J. Biol. Chem.* 246:6328–6334.

37. Francis, C. W., R. E. Markham, Jr., and V. J. Marder. 1984. Demonstration of in situ fibrin degradation in pathologic thrombi. *Blood.* 63:1216–1224.

38. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

39. Hoyer, L. W., and J. R. Shainoff. 1980. Factor VIII-related protein circulates in normal human plasma as high molecular weight multimers. *Blood.* 55:1056–1059.

40. Doolittle, R. F., K. W. K. Watt, B. A. Cottrell, D. D. Strong, and M. Riley. 1979. The amino acid sequence of the α-chain of human fibrinogen. *Nature (Lond.).* 280:464–468.

41. Semeraro, N., D. Collen, and M. Verstraete. 1977. On the origin of the Aα chain heterogeneity of human fibrinogen. *Biochim. Biophys. Acta.* 492:204–214.

42. Hoyer, L. W. 1981. The factor VIII complex: structure and function. *Blood.* 58:1–13.

43. Bohn, H. 1973. Comparative studies on the fibrin-stabilizing factors from human plasma, platelets and placenta. *Ann. N. Y. Acad. Sci.* 202:256–272.

44. Rodeghiero, F., T. Barbui, A. Dal Belin-Peruffo, and E. Dini. 1984. Defective fibrin crosslinking in acute leukemia. *Thromb. Haemostasis.* 52:343–346.

45. Shen, L. L., J. Hermans, J. McDonagh, R. P. McDonagh, and M. Carr. 1975. Effect of calcium ion and covalent cross-linking on formation and elasticity of fibrin gels. *Thromb. Res.* 6:255–265.

46. Gladner, J. A., and R. Nossal. 1983. Effects of crosslinking on the rigidity and proteolytic susceptibility of human fibrin clots. *Thromb. Res.* 30:273–288.

47. Urokinase Pulmonary Embolism Trial Group. 1970. Urokinase pulmonary embolism trial. Phase 1 results. A cooperative study. *JAMA (J. Am. Med. Assoc.).* 214:2163–2172.

48. Marder, V. J., R. L. Soulen, V. Atichartakarn, A. Z. Budzynski, S. Parulekar, J. R. Kim, N. Edward, J. Zahavi, and K. M. Algazy. 1977. Quantitative venographic assessment of deep vein thrombosis in the evaluation of streptokinase and heparin therapy. *J. Lab. Clin. Med.* 89:10-18–1029.