Influence of a Natural and a Synthetic Inhibitor of Factor XIIIa on Fibrin Clot Rheology

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ABSTRACT We investigated the origins of greater clot rigidity associated with FXIIIa-dependent cross-linking. Fibrin clots were examined in which cross-linking was controlled through the use of two inhibitors: a highly specific active-center-directed synthetic inhibitor of FXIIIa, 1,3-dimethyl-4,5-diphenyl-2[2(oxopropyl)thio]imidazolium trifluoromethylsulfonate, and a patient-derived immunoglobulin directed mainly against the thrombin-activated catalytic A subunits of thrombin-activated FXIII. Cross-linked fibrin chains were identified and quantified by one- and two-dimensional gel electrophoresis and immunostaining with antibodies specific for the α- and γ-chains of fibrin. Gamma-dimers, γ-multimers, α_n-polymers, and $\alpha_p\gamma_q$-hybrids were detected. The synthetic inhibitor was highly effective in preventing the production of all cross-linked species. In contrast, the autoimmune antibody of the patient caused primarily an inhibition of α-chain cross-linking. Clot rigidities (storage moduli, G') were measured with a cone and plate rheometer and correlated with the distributions of the various cross-linked species found in the clots. Our findings indicate that the FXIIIa-induced dimeric cross-linking of γ-chains by itself is not sufficient to stiffen the fibrin networks. Instead, the augmentation of clot rigidity was more strongly correlated with the formation of γ-multimers, α_n-polymers, and $\alpha_p\gamma_q$-hybrid cross-links. A mechanism is proposed to explain how these cross-linked species may enhance clot rigidity.

INTRODUCTION

The stabilization of fibrin by activated factor XIII (FXIIIa) produces remarkable effects on the rheological properties of clots. This enzymatic process of ligation, often inappropriately referred to in the biochemical literature as cross-linking (Lorand, 1972), results from the FXIIIa-catalyzed formation of N^{ϵ}-(γ-glutamyl)lysine isopeptide bonds between fibrin molecules within clot fibers (Lorand et al., 1968; Matacic and Loewy, 1968; Pisano et al., 1968). As shown in Ryan et al., 1999, the architecture of the fibers is essentially unchanged by these cross-links, though isolated chemical bonding between fibers that touch each other would not be visible with electron microscopy. Ligated clots exhibit mechanical stiffnesses up to five times greater than their unligated counterparts (Ferry et al., 1951; Roberts et al., 1973; Gerth et al., 1974; Mockros et al., 1974; Glover et al., 1975; Shen et al., 1975; Shen and Lorand, 1983) and do not display the stress-induced structural rearrangements seen in unligated clots by creep and by permanent deformation after creep recovery (Nelb et al., 1976, 1981; Janmey et al., 1983). The physiological significance of the FXIIIa-induced clot stabilization is demonstrated clinically by the severe hemorrhagic tendencies that are often exhibited by patients who suffer from a congenital absence of the factor XIII zymogen (Lorand et al., 1980) or who have a circulating inhibitor, frequently an autoimmune antibody, directed against some component of the factor XIII system in plasma (Lorand, 1994). Clots or experimental thrombi formed in whole human blood show significantly greater susceptibilities to lytic agents in the presence of competitive inhibitors of cross-linking (Lorand and Jacobsen, 1962; Bruner-Lorand et al., 1966; Lorand and Nilsson, 1972); the same effect can be brought about by blocking the enzymatic activity of FXIIIa by noncompetitive inhibitors (Samama et al., 1979; Freund et al., 1994) or by antibodies to factor XIII (Reed and Lukacova, 1995).

Although factor XIIIa is known to stabilize the clot network, the mechanism by which this cross-linking reaction augments clot stiffness remains largely undetermined. Ligations between Lys^{406} and Glu^{398} of the γ-chains of two adjacent fibrin molecules occur rapidly (Chen and Doolittle, 1971; Doolittle et al., 1971), resulting in the formation of γ-dimers (Chen and Doolittle, 1969; McKee et al., 1970) that could be oriented either longitudinally end-to-end between fibrin molecules (Fowler et al., 1981; Weisel et al., 1993; Veklich et al., 1998) or transversally across molecules of different fibrin strands (Selmayr et al., 1985, 1988; Mosesson et al., 1989, 1995; Siebenlist et al., 1995). Multiple cross-linking between fibrin α-chains results in the formation of α-polymers (McKee et al., 1970; Schwartz et al., 1971; Sobel et al., 1988; Gron et al., 1992), creating potentially intricate and, as yet, ill-defined arrangements between fibrin molecules. The enhancement of rigidity in ligated clots has been attributed mainly to the development of α-polymers, whereas γ-dimerization is thought to have a smaller (Glover et al., 1975; Gladner and Nossal, 1983) or even a negligible (Shen et al., 1974, 1975) effect on clot

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mechanical stiffness. Small percentages of FXIIIa-induced hybrid $\alpha_p\gamma_q$-ligations (Mosesson et al., 1989; Murthy and Lorand, 1990; Shainoff et al., 1991; Gron et al., 1993) and higher-order γ-chain ligations (γ-trimers and -tetramers; Mosesson et al., 1989; Murthy and Lorand, 1990; Shainoff et al., 1991; Siebenlist and Mosesson, 1992) have also been observed, but their contributions to clot rigidity have not previously been considered.

The present investigation was undertaken to study the effects of the individual ligated species on clot mechanical behavior. Cross-link formation in fibrin clots was controlled by using two inhibitors: a highly specific, active-center-directed synthetic inhibitor of FXIIIa, 1,3-dimethyl-4,5-diphenyl-2[(2-oxopropyl)thio]imidazolium trifluoromethylsulfonate (Freund et al., 1994), and a patient-derived immunoglobulin directed mainly against the thrombin-activated catalytic A subunits of FXIII (Green et al., 1992; Lorand et al., 1999). The autoimmune antibody, which had been implicated in the patient's recurrent hematomas, inhibited mainly the ligating of α-chains but not γ-chains. By monitoring clot rigidity with a cone and plate rheometer and evaluating the production of the ligation species of fibrin chains by one- and two-dimensional gel electrophoresis, a correlation was sought between the biochemical events catalyzed by FXIIIa and the development of clot rigidity.

MATERIALS AND METHODS

Human fibrinogen (plasminogen free; American Diagnostica, Greenwich, CT) was dissolved by addition of water, then dialyzed against 4 l of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA at 4°C, and stored at −80°C. Protein concentration was determined by absorbance at 280 nm ($E^{1\%}_{1cm}$ = 15.1; Mihalyi, 1968). The commercial preparation was contaminated by small amounts of FXIII, as judged by the detection of bands of α_n and γ–γ cross-linked chains in reducing SDS-PAGE after clotting the fibrinogen (6 μM) with human α-thrombin (1 NIHU/ml) and $CaCl_2$ (5 mM) for 90 min at 37°C. Human α-thrombin (a gift from Dr. J. W. Fenton II, New York State Department of Health, Albany, NY) was diluted to 500 NIHU/ml in 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 and stored at −80°C.

Factor XIII was purified from outdated human CPDA-1 plasma according to the procedure of Lorand et al. (1981) and stored in 50 mM Tris-HCl, 1 mM EDTA, 10 KIU/ml Trasylol, pH 7.5 at 4°C. Protein concentration of the stock solution was determined by absorbance at 280 nm ($E^{1\%}_{1cm}$ = 13.8; Schwartz et al., 1973). Just before experiments, the factor XIII zymogen (0.33 mg/ml) was preactivated by treatment with human α-thrombin (2 NIHU/ml) in 50 mM Tris-HCl, pH 7.5 for 25 min at room temperature. The 1,3-dimethyl-4,5-diphenyl-2[(2-oxopropyl)thio]imidazolium trifluoromethylsulfonate (L683685, Merck, Sharp and Dohme Research Laboratories, West Point, PA), was dissolved in 50 mM Tris-HCl, pH 7.5 with 1% DMSO and stored at −20°C at a concentration of 0.5 mM.

Patient and normal IgG were prepared by Pauline Velasco from plasma by affinity chromatography (GammaBind Plus Sepharose, Pharmacia Biotech, Piscataway, NJ) according to manufacturer's directions. The IgG was dialyzed against 10 mM Tris-HCl, 154 mM NaCl, pH 7.5 at 4°C, concentrated using a Centricon-30 Concentrator (Amicon, Beverly, MA), and stored at 4°C. Protein concentrations were determined by absorbance at 280 nm ($E^{1\%}_{1cm}$ = 13.5).

Electrophoresis and immunostaining

Electrophoretic experiments were carried out on clots formed from mixtures with compositions identical to those used in the rheological studies. Clots were generated at 21°C or 37°C in 50–250 μl reaction mixtures that typically were comprised of 6 μM fibrinogen, 31 nM purified factor XIIIa supplement, 50 mM Tris-HCl (pH 7.5), 0.3% or 0.4% DMSO, 0–150 μM of the FXIIIa inhibitor, sufficient amounts of NaCl (from a stock of 200 mM) to maintain final ionic strengths within the 0.158–0.161 range, 5 mM $CaCl_2$, and 3.5 NIHU/ml thrombin. In samples prepared for one-dimensional SDS-PAGE, the ligating reaction was stopped by solubilization in 6 M urea, 40 mM dithiothreitol, and 2% SDS at 37°C for 45 min. Samples prepared with IgG were thoroughly washed with 25 mM EDTA to remove the IgG and centrifuged to a pellet before solubilization. Ligations in the reaction mixtures prepared for isoelectric focusing were stopped by boiling for 1 min in 5 M urea, 103 mM dithiothreitol, 5% 2-mercaptoethanol, 10% ampholine pH 3.5–10, and 2.9% Nonidet P-40, followed by exposure to 37°C for 5 h.

One-dimensional SDS-PAGE (8% acrylamide) was performed by the procedure of Laemmli (1970) in a Mini-Protean II Dual Slab Cell (BioRad, Hercules, CA). Samples of 6 μg protein per lane were analyzed, and a broad-range molecular weight standard (BioRad) was used for reference. Two-dimensional polyacrylamide gel electrophoresis was carried out according to the method of O'Farrell (1975). Isoelectric focusing in the first dimension was performed in a Mini-Protean II 2-D Cell (BioRad) with 4.5 μg protein samples in ampholine (1.6%) between pH 3.5 and 10. Separation in the second dimension was carried out by SDS-PAGE (8% acrylamide) as described above.

Electroblotting to nitrocellulose (0.2 μm pore size, Schleicher and Schuell, Keene, NH) was performed in a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) for 2.5 h at 4°C by the procedure of Towbin et al. (1979) in 25 mM Tris, 192 mM glycine, 20% methanol. Gels were stained with Coomassie brilliant blue R (0.025% in 10% acetic acid and 20% methanol), and amido black (1% in 10% acetic acid and 50% methanol) was used for staining the nitrocellulose transblots. Photographs of the gels and transblots in the figures show the protein bands with M_r values higher than 31,000.

A mouse hybridoma supernatant (Ta 7.1.1) raised (by Dr. Lin Gu of our Blood Cell Function and Development Program Project) against a tryptic fragment isolated from the α-chain of human fibrin, containing residues 291–348 (by Dr. Gennady Samokhin of Northwestern University), was used for identifying bands with α-chains. The supernatant was used in 1:50,000 dilution after one-dimensional electrophoresis and in 1:10,000 dilution after two-dimensional electrophoresis. Bands containing γ-chains were detected with a monoclonal antibody (6/20/5) against residues 392–406 of the fibrin γ-chain (a gift from Dr. Bohdan J. Kudryk, New York Blood Center; 1:40,000 dilution after one-dimensional electrophoresis, 1:9000 dilution after two-dimensional electrophoresis). Unbound sites on the nitrocellulose blots were blocked with 2% nonfat dry milk powder in 10 mM sodium phosphate, 150 mM NaCl, pH 7.5. An alkaline phosphatase-conjugated anti-mouse IgG (1:5000 dilution, Sigma, St. Louis, MO, A-1902) was used as secondary antibody, and binding to the mouse IgG was detected by application of 0.38 M nitroblue tetrazolium (Sigma) with 0.36 M 5-bromo-4-chloro-3-indolylphosphate (Sigma). To enhance contrasts, two-dimensional blots were reprobed with antibodies and developed by repeating the steps described above. Relative intensities of Coomassie blue-stained bands were measured in an LKB Ultroscan XL Laser Densitometer (Bromma, Sweden) and quantitated by excising and weighing the recorded peak areas.

Measurement of clot rigidity

A VOR Rheometer (Bohlin Rheologi, Cranbury, NJ) was used for measuring the rheological properties of clots. Bohlin VOR Rheometer Software Version 4.05 (Bohlin Reologi AB, Sweden) was used for data collection and for calculating viscoelastic parameters. Reaction components were mixed in a 1.5-ml Eppendorf tube with buffer (50 mM Tris-HCl, 100–150 mM NaCl, pH 7.5; 330 μl total volume and ionic strength of 0.158–0.161). After the addition of preactivated factor XIIIa and human α-thrombin, the last step for initiating coagulation, the reaction components were thoroughly mixed with a pipette tip, and 325 μl of the clotting mixture were

quickly transferred from the tube to the stainless steel rheometer cone and plate fixture (which had a 2.5° cone angle and 30 mm diameter). Measurements were taken at 0.1 Hz under an imposed strain of 0.015. For measuring purposes, the rheometer operated in an oscillatory mode, with data collected over 10-s periods every 2 min. Between the 2-min intervals the clot remained at rest. The rheological behavior of clots formed under oscillatory strains, as in these experiments, did not differ significantly from those of clots formed without imposed oscillation (data not shown). Samples were tested at 21°C or 37°C. To prevent dehydration, heavy mineral oil (Walgreen Co., Deerfield, IL) was applied to a ring surrounding the exposed surfaces of the clots at the beginning of experiments.

RESULTS

Effect of a patient's FXIII-directed IgG on clot rigidity and cross-linking

Reducing SDS-PAGE analysis of fibrinogen clotted with thrombin and calcium revealed that the amount of FXIII contaminating the fibrinogen preparation was sufficient to effect nearly full α- and γ-chain cross-linking after 2 h, resulting in the development of γ-dimers and several unidentified higher-molecular-weight cross-linked species labeled X1–X5 (Fig. 1, *lane 1*). The formation of these ligated species appeared unaffected by the addition of normal IgG (3 mg/ml, *lane 2*). Rheological analysis of samples of the same compositions as those analyzed by SDS-PAGE revealed that clot rigidity, expressed as storage modulus (G'), was slightly lower in the presence of normal IgG (90.6 $\pm$ 14.5 Pa) than in its absence (106.8 $\pm$ 14.2 Pa). In sharp contrast, the presence of 3 mg/ml of the patient-derived IgG caused a much larger drop in G' (to 49.8 $\pm$ 11.6 Pa; p = 0.003). Formation of several of the higher-molecular-weight cross-linked species, particularly bands X1–X3, was inhibited at this concentration of patient IgG, but γ-dimerization was not diminished (*lane 3*).

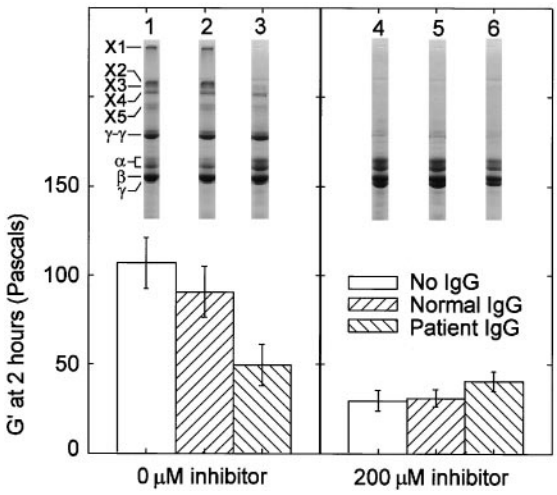

FIGURE 1 Effect of FXIII-directed IgG on clot rigidity and cross-linking. Clots were formed at 21°C from solutions of fibrinogen (6 μM), thrombin (3.5 NIHU/ml), $CaCl_2$ (5 mM), DMSO (0.4%), synthetic inhibitor of FXIIIa (0 or 200 μM), and IgG (0 or 3 mg/ml). Samples having the same compositions as those tested in the rheometer were analyzed by reducing SDS-PAGE.

More complete inhibition of cross-linking could be accomplished with the use of the synthetic inhibitor of FXIIIa, 1,3-dimethyl-4,5-diphenyl-2[2(oxopropyl)thio]imidazolium trifluoromethylsulfonate (Fig. 1, *lanes 4–6*). At a concentration of 200 μM, this inhibitor prevented the formation of all higher ligated species (X1–X5) and nearly all of the γ-dimers, regardless of whether the normal or the patient IgG was present. Nevertheless, despite nearly complete inhibition of cross-linking, average stiffnesses (29.7 $\pm$ 5.7 Pa without IgG, 31.1 $\pm$ 4.8 Pa with normal IgG, and 40.7 $\pm$ 5.4 Pa with patient IgG) were only slightly lower (p = 0.029, 0.033, and 0.144, respectively) than those of samples inhibited by the patient-derived IgG alone (49.8 $\pm$ 11.6 Pa).

Clot rigidities at various initial concentrations of fibrinogen: influence of the synthetic inhibitor of FXIIIa

The potency of the synthetic inhibitor is further demonstrated in its ability to significantly reduce the stiffness of clots over a wide range of physiological fibrinogen concentrations. Increasing the concentration of fibrinogen has been observed to increase clot rigidity in the presence (Samama et al., 1963; Kaibara, 1973; Glover et al., 1975; Gerth et al., 1974; Nelb et al., 1976) as well as in the absence of FXIIIa (Gerth et al., 1974; Nelb et al., 1976). Fig. 2 shows the dependence of clot stiffness on fibrinogen concentration under cross-linking conditions (*solid circles*) and the threefold reduction in clot rigidity produced at all fibrinogen concentrations in the presence of 2.5 μM of the inhibitor (*open circles*); e.g., in the presence of the inhibitor, the stiffness of the clot formed from an FXIIIa-containing mixture with a relatively high concentration of fibrinogen (9 μM) was reduced to that of a clot formed from a lower concentration of fibrinogen (3 μM) without the inhibitor.

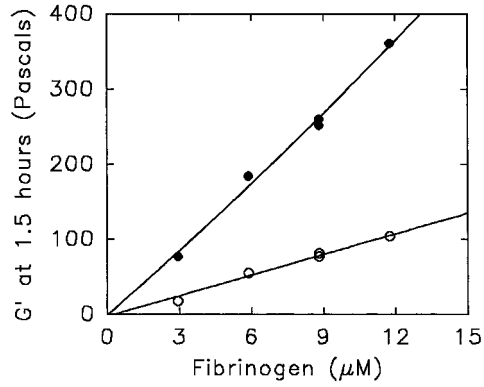

FIGURE 2 Effect of the active-center-directed inhibitor of FXIIIa, 1,3-dimethyl-4,5-diphenyl-2[2(oxopropyl)thio]imidazolium trifluoromethylsulfonate, on clot rigidity at various initial concentrations of fibrinogen. Clots were formed at 37°C with mixtures of fibrinogen (~3–12 μM), thrombin (0.5 NIHU/ml), $CaCl_2$ (5 mM), and FXIII (31 nM, *solid circles*) or inhibitor (2.5 μM, *open circles*); all mixtures contained 1% DMSO.

Profiles of ligated fibrin chains formed in the presence of the factor XIIIa inhibitor

The chain patterns of fibrin were examined by gel electrophoresis coupled with immunostaining to document the effects of the synthetic inhibitor on the FXIIIa-catalyzed reaction. Clots were formed at 37°C in the presence of varying concentrations of the inhibitor and examined by reducing SDS-gel electrophoresis after 2 h of clotting (Fig. 3 *A*). The fibrinogen control revealed a heterogeneity of the Aα-chains, consistent with cleavages near the carboxy-terminal regions (Mills and Karpatkin, 1970; Mosesson et al., 1972b). Three main monomeric Aα-species were observed in the M_r range of 52,000–67,000; immunostaining with an antibody to the α-chain (Fig. 3 *B*) also identified some Aα-chain degradation products near the position of Bβ- and β-chains and in some minor bands with M_r values lower than 47,000. A complete release of fibrinopeptides A and B occurred in the thrombin-catalyzed conversion of fibrinogen to fibrin, as demonstrated by the shifts of the Aα and Bβ bands to lower M_r values upon conversion to α and β chains (compare *lanes 1* and *2* in Fig. 3 *A*). The concentration of the FXIIIa used for the cross-linking reaction in these experiments was clearly sufficient to effect a full depletion of γ-monomers (*lane 3*). Two populations of ligated γ-dimers were observed, having electrophoretic mobilities compatible with the formation of γ–γ and γ'–γ' dimers (Francis et al., 1980; Lawrence et al., 1993). Ligated chains at the top of the stacking gel (marked as X1 in Fig. 3 *B*) are thought to comprise homologous α_n-polymers (McKee et al., 1970; Schwartz et al., 1971; Francis and Marder, 1987), but due to difficulties in transferring these chain types in the Western blotting experiments, their homologous polymeric nature could not be directly confirmed. Other high-molecular-weight ligated chains above 180 kDa (designated X2–X5 in Fig. 3 *B*), which reacted with both the anti-α and anti-γ antibodies, were also detected. The X5 ligated material appeared to be comprised of two separate bands: the higher-molecular-weight band stained only with the antibody to the γ-chains and the lower one only with the antibody to the α-chains.

Cross-linking of the α-chains was particularly sensitive to the action of the inhibitor (Fig. 3 *A*). Formation of the largest polymeric band X1 was completely abolished when the inhibitor was used at a molar ratio above 3:1 to the FXIIIa added (i.e., 0.1:0.03 μM, *lane 4*). Production of the cross-linked bands X2–X5 could be prevented with an inhibitor-to-enzyme ratio of 320:1 (i.e., 10 μM inhibitor, *lane 9*). Gamma-dimerization was not affected until the concentration of the inhibitor well exceeded 1 μM (i.e., an inhibitor-to-enzyme ratio >32:1), and >150 μM of the inhibitor (i.e., a 4800-fold molar excess) was required to reduce the intensity of the band of γ-dimers to that seen in the starting fibrinogen sample. Quantitative evaluations of the changes brought about by increasing the concentration of the FXIIIa inhibitor, as obtained from the relevant gel scans, are presented in Fig. 4.

The development of γ-dimers and bands X1–X5 as a function of time is presented in Fig. 5. The cross-linked species formed in the order of their molecular weights, with the smaller ligations having the fastest rates of appearance.

Compositions of the variety of ligated species were further examined by two-dimensional electrophoresis, using isoelectric focusing in the first dimension and SDS-PAGE in the second (Fig. 6). Protein staining of the noncross-linked fibrin control revealed two spots of differing pI values for the γ-chain, the weaker of which exhibited a higher molecular weight and probably corresponds to the γ'-chain, a variant of the γ-chain having an extended carboxy-terminal sequence and constituting ~7–16% of the total

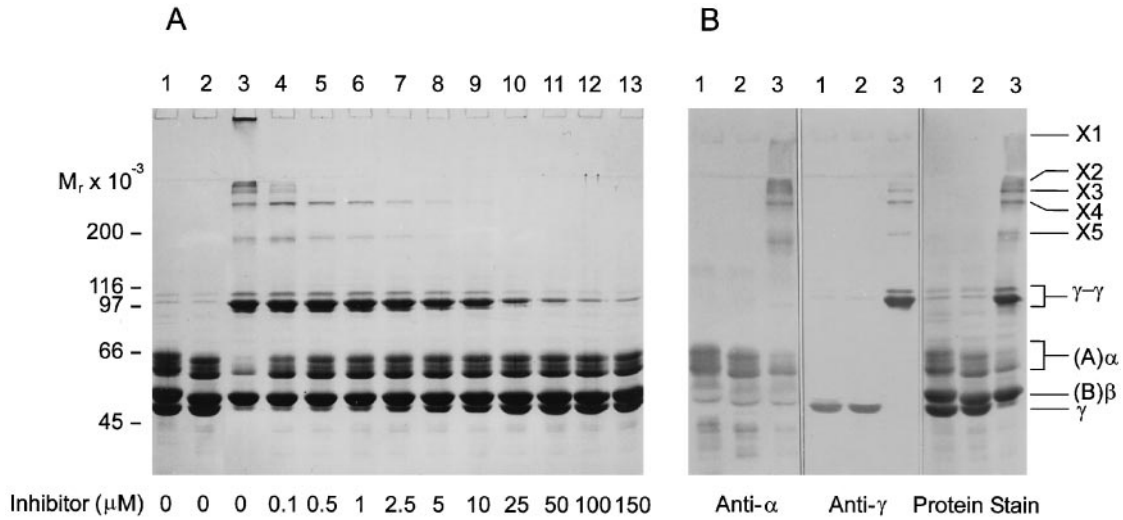

FIGURE 3 Changes in the ligated chain profile of fibrin at increasing concentrations of the synthetic inhibitor of FXIIIa. Clotting proceeded at 37°C for 2 h. The various mixtures (all containing 6 μM fibrinogen and 0.3% DMSO) were analyzed by reducing SDS-PAGE. (*A*) *Lane 1:* with EDTA (1 mM); *lane 2:* thrombin (3.5 NIHU/ml), FXIIIa (31 nM), and EDTA (1 mM); *lanes 3–13:* thrombin, FXIIIa, $CaCl_2$ (5 mM), and inhibitor (0–150 μM). (*B*) Immunostaining of the samples analyzed in lanes 1–3 of *A* revealed the presence of α- and γ-chain-related ligated polymers, marked X1–X5.

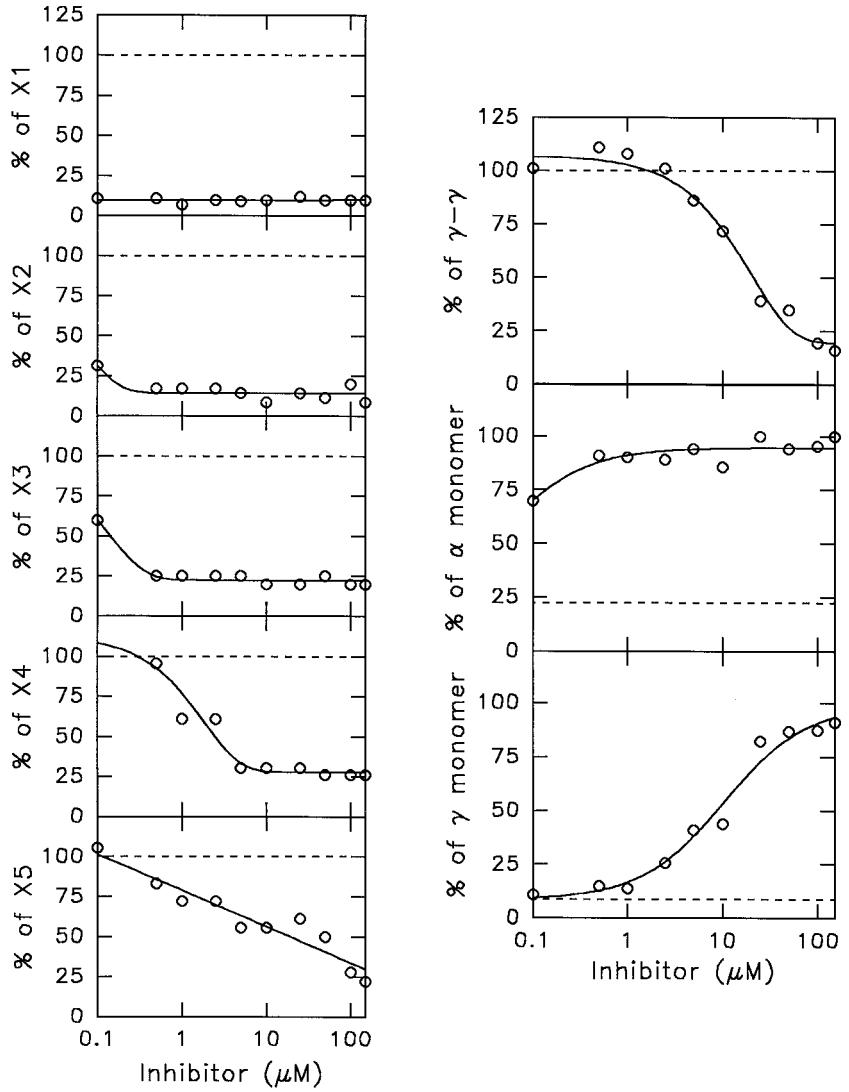

FIGURE 4 Densitometric scans of the lanes from Fig. 3 *A*. The percentages of ligated chains were calculated relative to those in lane 3 of Fig. 3 *A*. The percentages of α and γ monomers were calculated relative to those in lane 2. The amounts of monomers and cross-links were normalized to the percentages of β monomers in each lane. The percentages in the absence of inhibitor (lane 3) are represented by the broken lines.

γ-chain population in plasma (Mosesson et al., 1972a; Wolfenstein-Todel and Mosesson, 1980; Francis et al., 1980; Chung and Davie, 1984; Fornace et al., 1984). Multiple spots were observed for the α- and β-chains, consistent with the results of Teige et al., 1983; Carrell et al., 1983; Murthy and Lorand, 1990; and Gron et al., 1993. Immunostaining with the anti-α-chain antibody revealed several spots for the three major starting α-chain species and two for the α-chain derivatives at the β-chain position. Small amounts of γ-dimers were also present in the control fibrinogen.

Protein staining of the ligated fibrin transblot revealed the depletion of nearly all the monomeric γ and most of the major starting α-chains. The formation of two species of γ-dimers with pI values similar to those of the monomeric γ and γ' chains indicates homologous dimerization (i.e., the formation of γ–γ and γ'–γ' structures), which was also observed by Francis et al., 1980. Several higher-molecular-weight ligated polymers were seen with pI values similar to those of the dominant γ-dimer species and electrophoretic M_r values comparable to those of bands X2–X5 in Fig. 3 *B*. Inasmuch as these stained only with the γ-chain-specific antibody but not with the antibody to the α-chain, they seem to represent various homopolymers of γ-chains. The smallest of these polymers had an M_r value of a γ-tetramer. Two additional spots were detected with pI values slightly more alkaline than those of the homologous γ-polymers and with molecular masses comparable to those of bands X3 and X4 in Fig. 3 *B*. These spots reacted with antibodies to both the γ- and α-chains and could be identified as hybrid ligated chain products. Clearly, as indicated by the vertical streaks at the upper right-hand corners of the transblots, a number of other ligated species must have been produced in the reaction of fibrin with FXIIIa. These, however, failed to enter the gel during isoelectric focusing.

Effect of cross-link inhibition on fibrin clot rigidity

The rigidities (G') of fibrin clots having compositions corresponding to those examined by electrophoretic techniques

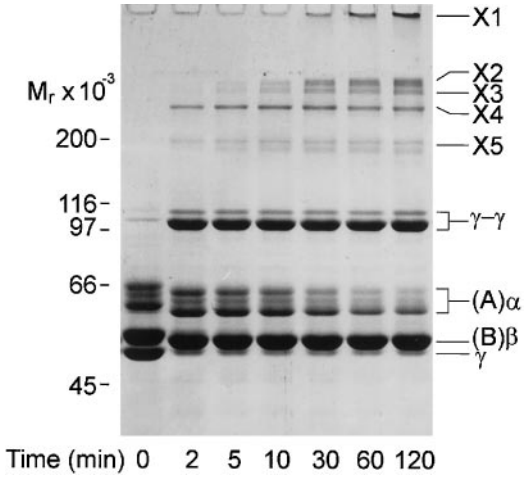

FIGURE 5 Development of ligated species with time. Reaction mixtures containing fibrinogen (6 μM), thrombin (3.5 NIHU/ml), $CaCl_2$ (5 mM), FXIIIa (31 nM), and DMSO (0.3%) were clotted at 37°C for various periods of time and analyzed by reducing SDS-PAGE.

in Fig. 3 were measured in the VOR Rheometer. With increasing concentrations of the FXIIIa inhibitor, the G' values exhibited by the clots (measured at 2 h of clotting) decreased from 135 Pa to a plateauing average of 45 Pa (Fig. 7). A minimum 32-fold molar excess of inhibitor over the added FXIIIa enzyme (i.e., 1 μM inhibitor) was required to reduce clot stiffness to this value.

The range of inhibitor concentrations that had the greatest effects on G' (0 to 1 μM) seems to best correlate with changes in the amounts of the higher-molecular-weight ligated species (bands X1–X5 in Fig. 3 *A*) but not with changes in the amounts of γ-dimer. Moreover, the high inhibitor concentrations (>1 μM) needed to prevent γ-dimer formation brought about no further decreases in G'. The relationship between the measured storage moduli of the clots (Fig. 7) and the amounts of the various ligated species as detected by SDS-PAGE (Fig. 4) is summarized in Fig. 8.

DISCUSSION

FXIIIa-induced clot stabilization produces no changes in the general appearance of fibrin networks observed under electron microscopy (Muller et al., 1984; Ryan et al., 1999) or in the mass-length ratios of fibers examined in turbidity experiments (Carr et al., 1987). The stiffening of clots produced by ligations, therefore, does not appear to originate from alterations in network morphology (i.e., the formation of new branchpoints or increased lateral aggregation), but most likely from the stiffening of the individual fibrin fibers already existing in the clot.

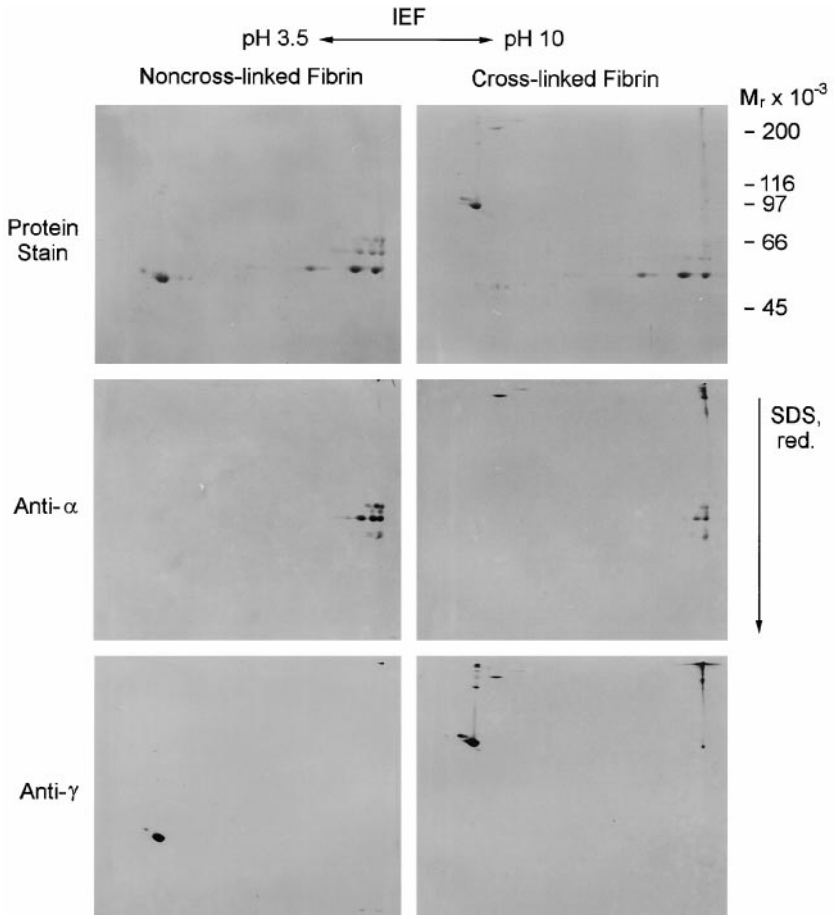

FIGURE 6 Analysis of fibrin chains by two-dimensional gel electrophoresis. Noncross-linked and cross-linked fibrin samples, of the same composition as those in lanes 2 and 3 of Fig. 3 *A*, were examined by protein staining and immunostaining with α- and γ-chain-specific antibodies.

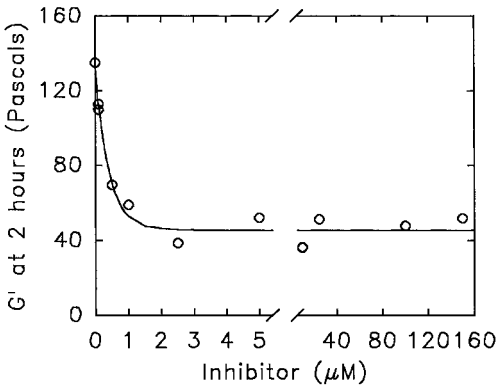

FIGURE 7 Effect of the synthetic inhibitor of FXIIIa on clot rigidity. Clots were formed at 37°C from solutions of fibrinogen (6 μM), thrombin (3.5 NIHU/ml), FXIIIa (31 nM), $CaCl_2$ (5 mM), DMSO (0.3%), and inhibitor (0–150 μM). Values for the storage modulus (G') were measured at 2 h of clotting.

The ability to control the formation of γ-dimers by use of the synthetic inhibitor of FXIIIa has enabled us to clearly demonstrate that γ-dimerization by the FXIIIa-catalyzed reaction, by itself, is not sufficient to enhance fiber stiffening. Previous investigators (Shen et al., 1974; 1975) had proposed the same idea but were unable to clearly demonstrate the effect of γ-dimerization on clot rigidity independent from the clot stiffening effects of calcium ions. The linkage of fibrin molecules by γ-chain dimerization takes place very early in clotting and may only initially function to secure the linear assembly of fibrin molecules without affecting protofibril rigidity. Ligations between γ-chains are believed to be oriented longitudinally between the ends of fibrin molecules (Fig. 9 *A*) or transversally between molecules of different fibrin strands (Fig. 9 *B*). Any bending or movement of double-stranded protofibrils between one another would theoretically not be restricted by the formation of longitudinally oriented γ-dimers alone (Fig. 9 *A*). Interprotofibrillar movement would also not be restricted by transverse cross-linking if dimeric γ-chain ligations were formed only between molecules of the same double-stranded protofibrils (Fig. 9 *B*). It is undetermined whether transverse ligations occur between different protofibrils (Fig. 9 *C*) or whether a combination of longitudinally oriented and transverse ligations are formed (Fig. 9 *D*). Any stiffening effects potentially imparted by the ligations of γ-dimers, however, may be dampened by the inherent flexibility of the fibrin molecule, which has been considered by several investigators (Cavazza et al., 1981; Hantgan, 1982; Weisel et al., 1987; Acuna et al., 1987; Hunziker et al., 1988; Montejo et al., 1992).

In our experiments, generation of ligated clot rigidity was closely related to the depletion of α-chain monomers and the development of higher-molecular-weight ligated species (X1–X5; Fig. 8). Augmentation of G' values was strongly associated with the formation of species X4 ($\alpha_p\gamma_q$-hybrids and possibly α_n-polymers) and, to a lesser degree, with that of X5 (γ-tetramers and α_n-polymers). The appearances of species X1 (of unknown composition), X2 (γ-multimers, possibly $\alpha_p\gamma_q$-hybrids and/or α_n-polymers), and X3 ($\alpha_p\gamma_q$-hybrids, γ-multimers, possibly α_n-polymers) coincided with

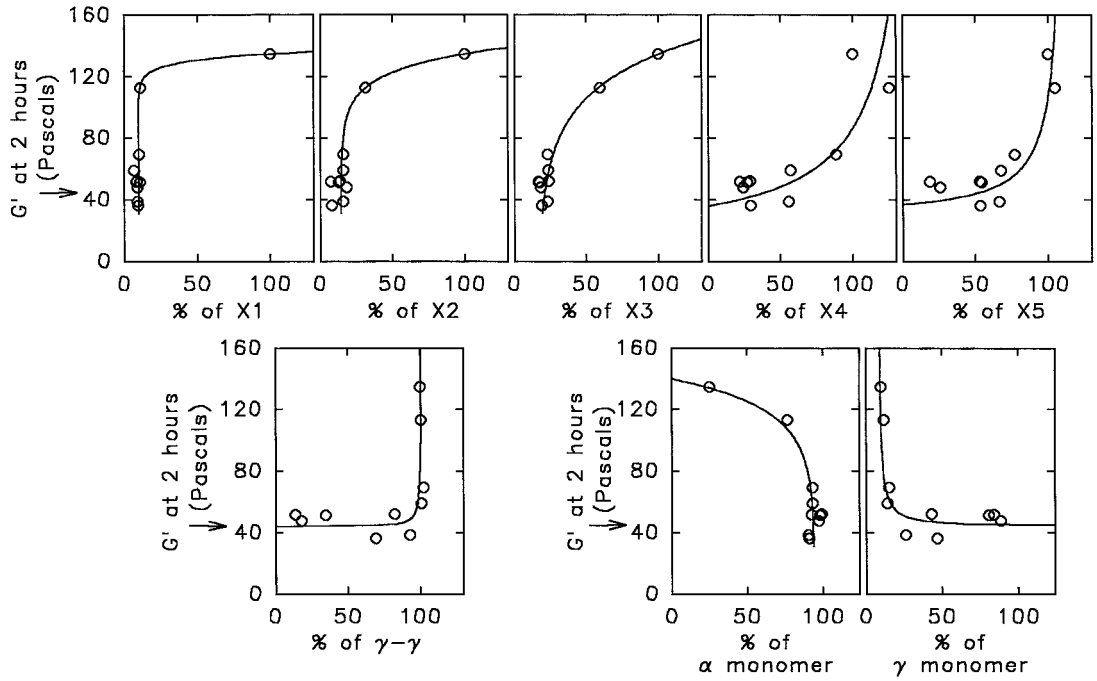

FIGURE 8 Effect of various ligated species on clot rigidity. The percentages of cross-linked species and monomeric fibrin chains found in clots analyzed by reducing SDS-PAGE (from Fig. 4) were correlated with the rigidities of clots of the same composition examined by rheometry (from Fig. 7). The stiffness of a nonligated clot was estimated from Fig. 7 to be ~45 Pa (*arrows*).

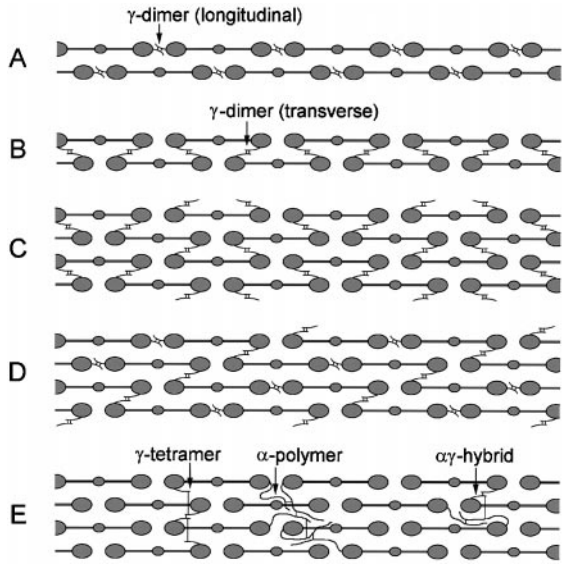

FIGURE 9 Schematic model of the possible orientations of ligated chains in fibrin protofibrils. (*A*) Longitudinal orientation of dimeric γ-chain ligations. (*B*) Transverse ligations of γ-dimers. In this diagram, transverse ligations occur only between molecules within the same double-stranded protofibril. (*C*) Transverse dimeric ligations of γ-chains between two adjacent protofibrils. (*D*) Longitudinal and transverse ligations of γ-chains between adjacent protofibrils. The patterns of ligations shown in *C* and *D* are only two of many that may potentially be formed between protofibrils arranged within a three-dimensional fibrin fiber. The γ-dimers in *A–D* are drawn with two ligations formed between the Lys^{406} and Glu^{398} of each γ-chain. It is undetermined, however, whether both or only one ligation is generated between the two γ-chains. The formation of only one ligation between the chains allows the possibility of the incorporation of the cross-linked γ-dimers into higher-order ligated species (i.e., γ-multimers and αγ-hybrid polymers). (*E*) The possible orientation of a γ-tetramer, α-polymer, and αγ-hybrid polymer between two adjacent protofibrils. For clarity, the γ-dimers have been omitted from this diagram.

increases in G' but did not seem to be necessary for the development of clot rigidities up to at least ~115, ~100, and ~70 Pa, respectively. The functional roles of these higher-molecular-weight ligated chain polymers in strengthening the fibrin network are possibly interdependent and would be difficult to separate from each other.

Further demonstration of the significance of the larger ligated chain types in enhancing clot rigidity is seen in the approximately twofold reduction in G' of clots in which the formation of species X1–X5 was inhibited by the FXIII-directed IgG (Fig. 1). The physiological importance of these ligated species is demonstrated by the hemorrhagic tendencies of the patient from whom the IgG was derived (Lorand et al., 1999). Despite the nearly complete ligation of γ-chains in clots formed in the presence of this antibody, the rigidity of clots formed with the IgG was only slightly higher than that of clots in which no γ-chain ligations were present. This small elevation of G' caused by the action of FXIIIa can most likely be attributed to the formation of low amounts of the X3–X5 species rather than the dimeric cross-linking of γ-chains.

The means by which the higher-order ligated species enhance fibrin network stiffness is unknown. Gamma-multimers (Fig. 9 *E*) are thought to form at fibrin network branchpoints or at sites of lateral fiber associations (Mosesson et al., 1989), and the increased network rigidity resulting from their development may point to the significance of fibrin strand thickness and branchpoint reinforcement in enhancing clot rigidity. The configuration of $\alpha_p\gamma_q$-hybrids and α_n-polymers among the fibrin fibers is potentially complex, but the mechanism by which the fibrin network is strengthened by these ligations could also be similar to that used by the γ-multimers. Unlike the cross-linked γ-dimers, these higher-order ligated chain species have the ability to form linkages between more than two molecules of a fiber and have the potential to secure several protofibrils together, thus restricting interfibril movement and decreasing fiber flexibility.

Although γ-chain dimerization by itself appears to have no direct affect on clot rigidity, the ligating of γ-chains is likely to provide the necessary structural framework for the later-developing, higher-order ligated chain species to enhance network stiffness. The production of ligated γ-dimers is believed to accelerate the ligating of $\alpha\gamma_2$-hybrid polymers (Shainoff et al., 1991) and is also thought to be necessary for the formation of γ-multimers (Siebenlist and Mosesson, 1992).

Rheological values in this investigation were measured in a relatively small strain regime (<0.1), in which changes in strain have little effect on clot structure or rigidity (Carr et al., 1976; Bale and Ferry, 1988; Janmey et al., 1983). At higher strains (>0.1), the possibility exists that the contributions of the γ-dimers and other cross-linked chain species to clot rigidity may be more significant. The roles of individual ligated species at these strains are, as yet, unknown.

In conclusion, our study demonstrates 1) the potential importance of the role of $\alpha_p\gamma_q$-hybrids, γ-multimers, and α_n-polymers in enhancing the ability of a clot to withstand mechanical stresses; 2) the absence of an effect on clot rigidity from dimeric γ-chain cross-linking alone; and 3) the effectiveness of the synthetic, active site-directed inhibitor of FXIIIa as an agent for controlling ligations. Elucidation of the structural origins of the FXIIIa-catalyzed cross-linking effects on clot rheological behavior is significant with regard to the expanding clinical use of rheological methods for evaluating the hemostatic status of patients (Zuckerman et al., 1981; Kang et al., 1985; Tuman et al., 1994; Ben-Ari et al., 1997; Kaufmann et al., 1997; Sharma et al., 1997).

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