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Protransglutaminase (Factor XIII) Mediated Crosslinking of Fibrinogen and Fibrin*

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Keywords

Factor XIII, fibrinogen, protransglutaminase, transglutaminase, familial mediterranean fever, α_2-antiplasmin

Summary

Plasma factor XIII (plasma protransglutaminase) circulates as an A_2B_2 tetramer bound to the γ' variant chains of fibrinogen "2". During clotting the A subunits of fXIII are cleaved by thrombin to form fXIIIa (transglutaminase) and in the presence of calcium ions, activated A_2* subunits dissociate from the B subunits. When purified plasma fXIII or recombinant cellular factor XIII (A_2) was incubated with fibrinogen in the presence of calcium ions (≥50 μM) a non-synerizing gel formed concomitant with formation of γ dimers, followed by Aγ polymers, and eventually γ trimers and γ tetramers. As is the case of fXIIIa, the fXIII-mediated crosslinking rate was enhanced in the presence of thiols. After an initial lag period, fXIII catalyzed fibrinogen crosslinking at ~75% of the rate of fXIIIa under typical crosslinking conditions (100 Loewy u/ml, 5 mM $CaCl_2$ & 500 μM DTT). Fibrin was crosslinked about 8 times more rapidly by fXIII than was fibrinogen, and after an initial lag period fXIII crosslinked fibrin at nearly the same rate as fXIIIa. Substituting plasma for purified fXIII as the source for fXIII resulted in robust fibrinogen crosslinking activity. In contrast to the high level of fXIII-mediated crosslinking activity observed with fibrinogen or fibrin as substrates, when transglutamination was measured using cadaverine incorporation into casein, fXIII was 30-fold less active than fXIIIa. Thus, factor XIII displays constitutive enzymatic activity with respect to fibrinogen and fibrin. The results further indicate that uncleaved fXIII in plasma provides a potent source of readily available crosslinking activity in clotting blood. Fibrinogen 2, whose γ'chains bind fXIII B subunits, was crosslinked 3.5 times more slowly by fXIII than was fibrinogen 1 (lacking γ' chains), suggesting that complex formation between fibrinogen 2 and plasma fXIII plays a significant role in down-regulating potential plasma fXIII-mediated crosslinking activity. Since fibrin is a considerably better substrate for fXIII than is fibrinogen, the rate at which crosslinking takes place in a fibrinogen-containing plasma environment is much lower than it would be if fibrin were present.

Introduction

Fibrinogen is a tridomainal disulfide linked protein comprised of two symmetrical halves, each composed of three polypeptide chains termed Aα, Bβ, and γ (1-3). Human fibrinogen can be separated by ion exchange chromatography into two major fractions, fibrinogen 1 ("peak 1 fibrinogen") and fibrinogen 2 ("peak 2 fibrinogen") (4, 5). The two fibrinogens differ from each other with respect to the composition of their γ chains. Fibrinogen 1 contains two γ_A chains which are comprised of 411 amino acids. Heterodimeric fibrinogen 2 molecules each contain one γ_A and one γ' chain (6, 7). The variant γ' chain is longer (427 residues), and has a more anionic, carboxyl terminal sequence than the γ_A chain beyond position 408 (8). Factor XIII has been shown to bind to the γ' chains in fibrinogen 2 (9) and thrombin has also been shown to bind to the same anionic γ' extension of fibrin 2 (10).

Following the rapid thrombin-mediated cleavage of fibrinopeptide A (11-15) and slower cleavage of fibrinopeptide B (16-18), fibrin assembly commences with formation of double stranded twisting fibrils in which fibrin molecules are arranged in a staggered overlapping manner (19-26). Subsequently, lateral fibril associations occur, resulting in thick fibers (22, 26-28). Concomitant with converting fibrinogen to fibrin, thrombin activates factor XIII to factor XIIIa. In the presence of fXIIIa and Ca^{2+}, fibrin undergoes intermolecular covalent crosslinking by formation of ε–amino(γ–glutamyl) lysine isopeptide bonds (29, 30). Crosslinking of γ chains within fibrils occurs between properly aligned chains at lysine 406 of one γ chain, and a glutamine at position 398 or 399 of another to form γ dimers (31, 32). Intermolecular crosslinking between α chains creates oligomers and larger α chain polymers and occurs more slowly than γ dimerization (32, 33). Gamma trimers and γ tetramers form after more prolonged incubation (19). Factor XIIIa can also crosslink fibrinogen, albeit at a somewhat slower rate (34, 35). Crosslinked fibrinogen forms a translucent, non-synerizing gel, that is associated with γ dimer formation (34, 35).

Plasma factor XIII (protransglutaminase) is a noncovalent tetrameric protein complex composed of two pairs of subunit chains termed "A" and "B", respectively (36-38). Thrombin cleavage at position 37 of the A subunits (39-41) leads to formation of the active enzyme, factor XIIIa (transglutaminase), which itself requires Ca^{2+} for dissociation of the activated subunits (A_2*) from the B subunits and for full expression of its catalytic activity (42-46). Activation of fXIII and the activity of

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[1] *Abbreviations:* fXIII, plasma factor XIII (A_2B_2); fXIIIa, factor XIIIa; cellular factor XIII, the homodimeric form of factor XIII (A_2) found in cells like platelets and placenta; DTT, dithiothreitol; Tris, tris(hydroxymethyl) aminomethane; β-MSH, β-mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; NEM, N-ethylmaleimide; FMF, Familial Mediterranean Fever.

fXIIIa is promoted in the presence of fibrinogen (45-51) and perhaps even more specifically, by fibrin (47-54). Inhibition of fibrin polymerization with the peptide GPRP, eliminates the enhancing effect of fibrin on fXIII activation (49, 52, 55), suggesting that the fibrin effect is mediated through formation of a ternary complex among thrombin, fibrin, and factor XIII (50).

Factor XIII can be activated independently of thrombin. Calcium ion concentrations greater than 100 mM activate fXIII and expose the active site cysteine to sulfhydryl modification (45). Chaotropic anions, such as *p*-toluenesulfonate or thiocyanate, reduce the calcium ion requirement for thrombin-independent activation of factor XIII to about 50 mM (56). Blombäck et al. (57) also noted that the fXIII "zymogen" in the presence of a supraphysiological Ca^{2+} concentration (20 mM) plus a reducing agent caused gelation of fibrinogen, suggesting that fXIII had intrinsic crosslinking activity. During the course of earlier studies on the interaction between fibrinogen 2 and factor XIII (9), we observed that mixtures of fibrinogen and fXIII formed gels when exposed to near physiological concentrations (5 mM) of Ca^{2+} (unpublished observations). In this present study those preliminary observations have been extended by thoroughly characterizing the crosslinking activity of the plasma factor XIII "zymogen" (A_2B_2) as well as that of the cellular form of factor XIII (A_2).

Materials and Methods

Tris1, glycine, Coomassie Brilliant Blue R250, N-ethylmaleimide, and DTT were purchased from Aldrich Chemical Co., Milwaukee, WI. Trasylol (aprotinin) was obtained from Miles Inc., Kankakee, IL. ^{14}C-cadaverine and N,N'-dimethylcasein was acquired from Sigma Chemical Co., St. Louis, MO, and DE-52 cellulose was from Whatman Inc., Clifton, NJ. Human α thrombin (3188 u/mg) was obtained from Enzyme Research Laboratories, South Bend, IN. Recombinant cellular factor XIII (A_2) was the kind gift of Paul Bishop (ZymoGenetics, Seattle, WA) and had a specific activity of 1550 Loewy u/mg when assayed as thrombin activated fXIIIa. Other chemicals were the highest purity available from commercial sources.

Human fibrinogen was isolated from pooled citrated plasma by glycine precipitation (58), and further purified as previously described (59). I 2 fibrinogen was subfractionated into fibrinogen 1 (γ_A-γ_A) and fibrinogen 2 (γ_A-γ') by chromatography on DE-52 (9). Fibrinogen 1 is factor XIII free, since all of the contaminating plasma fXIII elutes with fibrinogen 2 (9). The fibrinogen 2 used in these studies contained less than 5 Loewy units of fXIII activity per milligram of total protein. Soluble fibrin monomer was prepared by the method of Belitser et al. (60). Fibrinogen and soluble fibrin concentrations were determined spectrophotometrically at 280 nm using an absorbance coefficient ($A_{1\,cm}^{1\%}$) of 15.1 (61).

Factor XIII was purified from pooled human plasma (62) and assayed on fXIII free fibrin substrates (prepared from fibrinogen 1) in the presence of 10 mM $CaCl_2$ as thrombin-activated fXIIIa as described by Loewy et al. (63). Factor XIII concentrations were determined spectrophotometrically at 280 nm using an extinction coefficient ($A_{1\,cm}^{1\%}$) of 13.8 (36). The specific crosslinking activities of the thrombin-activated fXIII preparations were between 2100 and 2300 Loewy u/mg. The pooled human plasma (fibrinogen level, 2.83 mg/ml) used in these studies contained 105 Loewy u/ml factor XIII activity and single donor plasmas range from 85 to 120 u/ml. Purified factor XIII zymogen (loaded at 20 μg/band/lane) was resolved into two bands by non-reducing SDS-PAGE (64), the A subunit of 75 kD and the B subunit of 79 kD (36, 65). No active A subunit (A*; 72 kD) was detected in these preparations (sensitivity <0.1 μg/band/lane or less than 0.5% of the A subunit population). XIII was activated to XIIIa by incubation with thrombin (10 u/ml) (34) for 30 min at 37° C and the thrombin then inactivated by incubating with a 5 fold excess of hirudin (50 u/ml) (35). SDS-PAGE of XIIIa revealed two bands, the active A* subunit (72 kD) and the B subunit (79 kD) (36, 65).

Typical crosslinking reaction mixtures contained fibrinogen 1 (3 mg/ml) in 50 mM Tris, 100 mM NaCl, 5 mM $CaCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 10 KIU/ml Trasylol, pH 7.4. Crosslinking was initiated by adding fXIII (100 Loewy u/ml; 0.124 μM) and incubating at room temperature. The crosslinking conditions were modified for certain experiments by varying the fXIII concentration (0 to 200 Loewy u/ml), the calcium ion concentration (0 to 10 mM), the DTT concentration (0 to 5 mM), by employing different reducing agents (β-mercaptoethanol or cysteine), by using fibrinogen 2, by using soluble fibrins, by using recombinant cellular fXIII (A_2) (100 Loewy u/ml; 0.43 μM), or by using plasma (105 Loewy u/ml) as the source of the fXIII. The crosslinking reaction was terminated by adding 10 volumes of 2% acetic acid an incubating at room temperature for 1 h. Insoluble material was removed by centrifugation and the fibrinogen remaining in solution was quantified spectrophotometrically at 280 nm. These data were plotted and rate of crosslinking (% insoluble material/min) was calculated from the steepest slope of these plots. Alternatively, the reactions were terminated by adding an equal volume of 2X Laemmli sample buffer with 1% β-mercaptoethanol, and the products of the reaction were analyzed by SDS-PAGE employing the discontinuous buffer system of Laemmli (64) on 9% polyacrylamide gels. Gels were stained with 0.5% Coomassie Brilliant Blue R250. In certain control experiments NEM or iodoacetamide (0.5 mM final concentration) was added to inhibit fXIII or fXIIIa activity (57). Some crosslinking reactions were carried out at room temperature for up to 5 days. The resulting gels were washed, digested with plasmin (0.2 caseinolytic u/ml, final) and the digestion products were analyzed for D trimer and D tetramer by SDS-PAGE (19, 66).

Factor XIII or XIIIa activity was also determined using N,N'-dimethylcasein and ^{14}C-cadaverine as substrates as described by Lorand et al. (67). Briefly, N,N'-dimethylcasein (0.5%) and ^{14}C-cadaverine (1.5 mM) in 50 mM Tris, 100 mM NaCl, 5 mM $CaCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 10 KIU/ml Trasylol, pH 7.4 was mixed with various amounts of fXIII or fXIIIa (0 to 100 Loewy u/ml; 0 to 0.124 μM) and incubated at room temperature. At selected times 100 μl of the reaction mixture was spotted on Whatman 3MM filter paper and immediately plunged into ice cold 10% trichloroacetic acid. The filter papers were washed twice with ice cold 5% trichloroacetic acid, once with ethanol:acetone (1:1), and then with ice cold acetone. After air drying the sample radioactivities were counted.

The possible release of fibrinopeptides and the factor XIII activation peptide during the crosslinking reaction was examined by HPLC (46) using a Varian Vista model 5000 system controlled by a Varian model 401 microprocessor. Fibrinogen (3 mg/ml) in 50 mM Tris, 100 mM NaCl, 5 mM $CaCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 10 KIU/ml Trasylol, 0.1% PEG 6000, pH 7.4. The reaction was initiated by adding fXIII (850 Loewy u/ml; 1.0 μM) and the mixture was incubated at room temperature. Control reactions were initiated by the addition of fXIII (850 Loewy u/ml) and thrombin (0.05 u/ml). At selected intervals the reaction was terminated by incubation in a boiling water bath for 10 min. The samples were clarified by centrifugation and applied to an Altex C18 reverse-phase column (0.46 × 25 cm; Rainin Instruments Co, Woburn, MA) equilibrated with 90% 0.083 M sodium phosphate, pH 3.1, 10% acetonitrile (buffer A). Elution was performed isocratically for the first 10 min using a mixture of 85% buffer A/15% buffer B (buffer B; 60% 0.083 M sodium phosphate, pH 3.1, 40% acetonitrile) followed by a linear gradient from 85% buffer A/15% buffer B to 10% buffer A/90% buffer B over 50 min. Peptides were detected with a Waters detector set at 205 nm.

Results

The time course of plasma factor XIII-mediated crosslinking of fibrinogen is presented in Fig. 1. Gamma dimers are formed in the reaction mixture within 5 min of incubation at room temperature and α-polymers begin to appear at 10 min. Under the conditions employed, complete γ chain crosslinking required incubation times of greater than 12 h. D trimers and D tetramers, the markers for γ trimer and γ tetramer formation, could be detected in plasmic digests of fXIII crosslinked

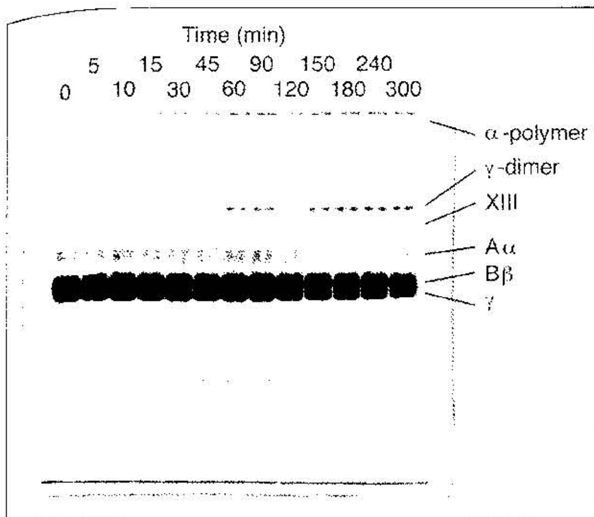

Fig. 1 Time course of plasma factor XIII-mediated crosslinking of fibrinogen. Fibrinogen I (3 mg/ml) and fXIII (100 Loewy u/ml) were incubated for the indicated times as described in Materials and Methods. Reactions were terminated by adding gel sample buffer containing 1% β-mercaptoethanol, and the products were separated by SDS-PAGE

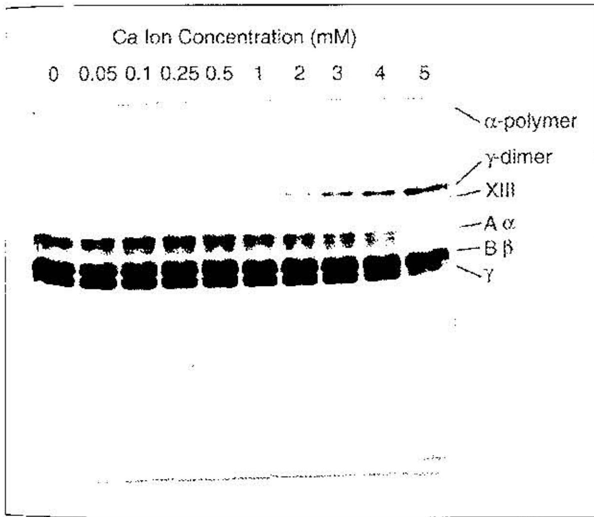

Fig. 2 Calcium ion sensitivity of fibrinogen crosslinking by plasma fXIII. Fibrinogen I (3 mg/ml) and fXIII (100 Loewy u/ml) were incubated as described in Materials and Methods with the indicated concentrations of $CaCl_2$. Reactions were terminated by adding gel sample buffer containing 1% β-mercaptoethanol, and the products were separated by SDS-PAGE

fibrinogen gels as early as 24 h into the incubation period and their concentrations increased with increasing times of incubation (data not shown). Crosslinking and gel formation was not due to contaminating thrombin activating the proteins since neither fibrinopeptide A or B nor the fXIII activation peptide could be detected in the reaction mixtures or in the factor XIII preparations alone even when the concentration of reactants was increased 8.5-fold and the incubation was allowed to occur for 96 h (results not shown).

Table 1 Fibrinogen crosslinking at various concentrations of factor XIII

XIII Concentration	Percent Soluble Fibrinogen	
(Loewy u/ml)	@ 6 hrs	@ 24 hrs
0	100 ± 1.9	100 ± 2.9
25	61.7 ± 3.3	13.9 ± 5.2
50	43.2 ± 2.3	8.3 ± 4.8
100	24.7 ± 3.8	5.7 ± 3.3
150	16.0 ± 2.4	4.7 ± 2.4
200	8.3 ± 4.3	3.8 ± 1.6

Fibrinogen 1 (3 mg/ml) and the indicated level of XIII in 50 mM Tris, 100 mM NaCl, 5 mM $CaCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 10 KIU/ml Trasylol, pH 7.4 buffer were incubated at room temperature. The reaction was terminated by the addition of 2% acetic acid, insoluble material removed by centrifugation, and the fibrinogen remaining in solution quantified spectrophotometrically at 280 nm. Mean ± standard deviation of four independent trials

The amount of fibrinogen incorporated into insoluble gels was directly proportional to the amount of fXIII present in the reaction mixture (Table 1). Calcium ions are absolutely required for fXIII-mediated crosslinking of fibrinogen, since in the absence of calcium no crosslinking was detected even after 24 h (Fig. 2). Concentrations of Ca^{2+} as low as 50 μM were sufficient to produce an easily measurable degree of γ chain crosslinking and the amount of γ dimer formation increased with increasing Ca^{2+} concentrations. Alpha polymers were detected in the reaction mixtures at Ca^{2+} concentrations above 1 mM. Optimal fibrinogen crosslinking was achieved at 24 h at 5 mM Ca^{2+} or higher.

Thiol reagents, though not required for plasma fXIII-mediated fibrinogen gelation, nevertheless increased the amount of fibrinogen rendered insoluble (Table 2). Adding DTT at a concentration of 2.5 μM increased the amount of fibrinogen incorporated into the gel five-fold and >80% gelation was achieved at 500 μM DTT. β-Mercaptoethanol

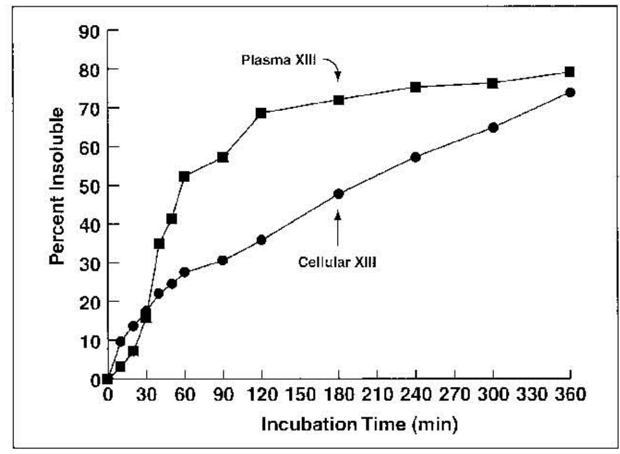

Fig. 3 Fibrinogen crosslinking by plasma fXIII (A_2B_2) or recombinant cellular fXIII (A_2). Fibrinogen 1 (3 mg/ml) was incubated for the indicated times with plasma fXIII (100 Loewy u/ml) or with recombinant cellular fXIII (100 Loewy units/ml) as described in Materials and Methods. Reactions were terminated by adding acetic acid and the fibrinogen remaining in solution was quantified spectrophotometrically. (■) plasma fXIII; (●) recombinant cellular fXIII. Mean of 6 independent experiments

Table 2 Thiol enhancement of plasma XIII-mediated fibrinogen crosslinking

Ca^{2+}, mM	Thiol, μM	Percent Soluble Fibrinogen		
		DTT	β-MSH	Cysteine
0	0	100 ± 2.2	100 ± 2.9	100 ± 1.8
4	0	94.4 ± 0.8	94.1 ± 0.3	95.8 ± 0.8
4	2.5	74.3 ± 3.9	88.6 ± 2.0	94.2 ± 0.5
4	5	64.6 ± 7.5	77.5 ± 5.4	93.9 ± 2.7
4	10	53.7 ± 6.8	71.7 ± 3.4	86.0 ± 2.0
4	25	46.3 ± 8.2	64.2 ± 4.1	78.7 ± 3.0
4	50	39.0 ± 8.2	55.3 ± 2.3	72.2 ± 3.9
4	100	33.7 ± 8.6	49.7 ± 5.0	64.1 ± 3.3
4	250	22.3 ± 4.5	42.7 ± 5.2	57.8 ± 2.5
4	500	18.5 ± 2.1	36.4 ± 5.3	53.9 ± 2.5
4	1000	15.6 ± 8.6	32.1 ± 2.0	51.4 ± 2.6

Fibrinogen 1 (3 mg/ml) and XIII (100 Loewy u/ml) in 50 mM Tris, 100 mM NaCl, 0.5 mM PMSF, 10 KIU/ml Trasylol, pH 7.4 buffer, were incubated with the indicated concentrations of Ca^{2+} and thiol reagent for 6 h at room temperature. The reaction was terminated by the addition of 2% acetic acid, insoluble material removed by centrifugation, and the fibrinogen remaining in solution quantified spectrophotometrically at 280 nm. Mean ± standard deviation of six independent trials

and cysteine also caused an increase in the amount of insoluble fibrinogen, but they were 2 to 3 fold less effective than DTT at any given concentration.

The kinetics of crosslinking by recombinant cellular factor XIII (A_2) were somewhat different than with plasma fXIII (Fig. 3). There was a lag in the initial rate of fibrinogen crosslinking by plasma fXIII not observed with cellular fXIII, suggesting that the presence of B subunits had retarded the onset of plasma fXIII-mediated crosslinking. Nevertheless, the crosslinking rate with plasma fXIII eventually surpassed that of cellular fXIII, and at its maximum was twice that of recombinant cellular fXIII, again suggesting that the non-catalytic B subunits had modified the crosslinking activity of the A_2 subunits.

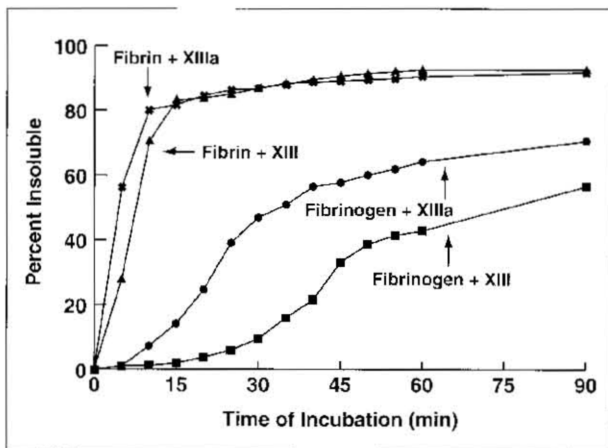

Fig. 4 Fibrinogen or fibrin crosslinking by plasma fXIII or fXIIIa. Fibrinogen 1 or solubilized fibrin 1 (3 mg/ml) and fXIII or thrombin activated fXIIIa (100 Loewy u/ml) were incubated for the indicated times as described in Materials and Methods. Reactions were terminated by adding acetic acid and the fibrinogen remaining in solution was quantified spectrophotometrically. (■) fibrinogen & fXIII; (●) fibrinogen & fXIIIa; (▲) fibrin & fXIII; (X) fibrin & fXIIIa. Mean of 6 independent experiments

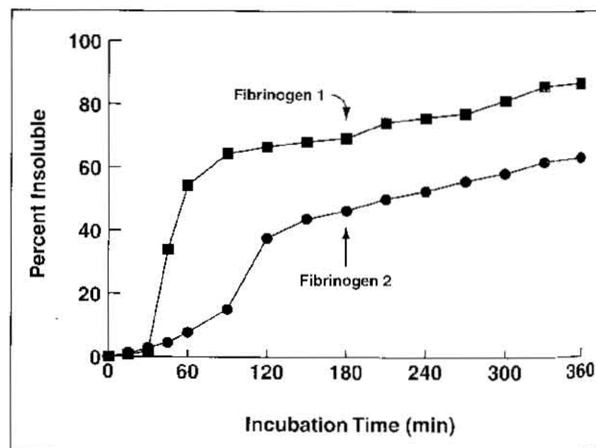

Fig. 5 Fibrinogen 1 or fibrinogen 2 crosslinking by plasma fXIII. Fibrinogen 1 or fibrinogen 2 (3 mg/ml) and fXIII (100 Loewy u/ml) were incubated for the indicated times as described in Materials and Methods. Reactions were terminated by adding acetic acid and the fibrinogen remaining in solution was quantified spectrophotometrically. (■) fibrinogen 1; (●) fibrinogen 2. Mean of 4 independent experiments

The rate of crosslinking catalyzed by plasma fXIII and thrombin-activated fXIIIa are compared in Fig. 4. With fibrinogen as substrate, factor XIII displayed a longer lag period than did fXIIIa, but eventually its rate approached 75% that of fXIIIa. The rate of fibrin crosslinking by factor XIII was almost 8 times faster than it was with fibrinogen, and the rate of fibrin crosslinking by fXIIIa was about 6 times faster than with fibrinogen. In the case of fibrin crosslinking, as had been the case with fibrinogen, factor fXIII exhibited an initial lag phase compared with fXIIIa, but eventually its crosslinking rate became virtually the same as that of fXIIIa.

The fact that fibrinogen 2 binds plasma factor XIII B subunits (9) suggested that non-covalent association of B and A subunits on fibrinogen 2 might function to suppress fXIII crosslinking activity. Indeed, when fibrinogen 2 was incubated with plasma fXIII the lag time of the crosslinking reaction was nearly twice as long as that observed with fibrinogen 1 and the maximum rate of fibrinogen crosslinking was decreased 3.5-fold (Fig. 5). This suggests that the complex formed between fibrinogen 2 and plasma fXIII effectively slowed the development of crosslinking activity of the catalytic A_2 subunits by slowing their release from the complex.

The relative rate of crosslinking by fXIII and fXIIIa was very different with N,N'-dimethylcasein as a substrate compared with fibrinogen or fibrin (Fig. 6). At an activity level of 100 Loewy u/ml, thrombin-activated fXIIIa incorporated ^{14}C-cadaverine into casein at least 30 fold more rapidly than did the factor XIII zymogen, which had very little activity. These findings suggest that the robust transglutaminase activity displayed by of factor XIII is specific for fibrinogen or fibrin as substrates since it occurs very poorly with a non-physiological substrate like casein.

It seemed possible that a small amount of factor XIII had become activated during the purification process (albeit undetectable by SDS-PAGE). To assess this possibility, fibrinogen crosslinking with pooled human plasma as the fXIII source was compared with the purified plasma fXIII (Fig. 7). The reaction mixtures containing the plasma as the source of fXIII formed insoluble crosslinked fibrinogen at a faster rate and to a larger extent than did samples containing an equivalent potential activity of purified fXIII. The effect was more marked in the presence of 0.4 mM DTT (panel A) than it was at 0.05 mM DTT (panel B). Gelation was not attributable to thrombin activity in the plasma-containing samples since control mixtures in which the fXIII was inhibited by NEM or iodoacetamide (0.5 mM) did not form uncrosslinked clots during a 24 h incubation period (not shown). These results suggest that if anything, the purification process may lead to a reduction in the intrinsic crosslinking potential of factor XIII.

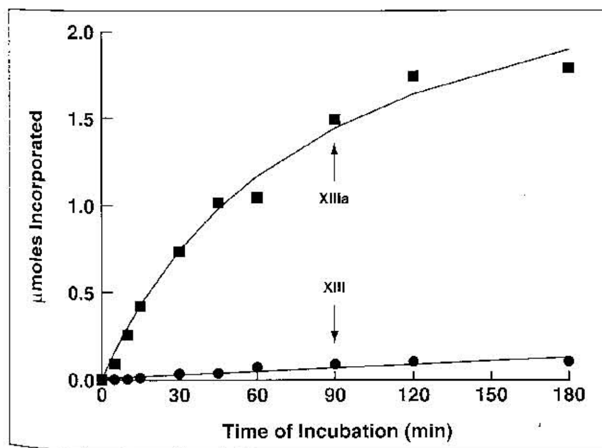

Fig. 6 ^{14}C-cadaverine incorporation into N,N'-dimethylcasein by plasma fXIII or fXIIIa. N,N' dimethylcasein (0.5%) and ^{14}C-cadaverine (1.5 mM) was mixed with fXIII or fXIIIa (100 Loewy u/ml) and incubated at room temperature. At selected times 100 µl of the reaction mixture was spotted on Whatman 3MM filter paper and treated as described in the methods section. (■) fXIIIa; (●) fXIII. Mean of 3 independent experiments

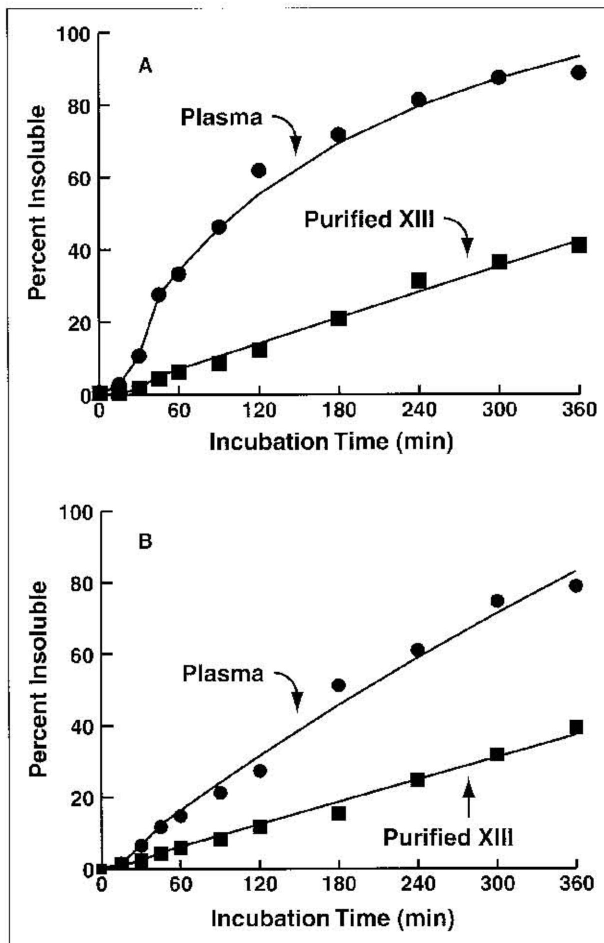

Fig. 7 Fibrinogen crosslinking mediated by purified factor XIII or plasma. Fibrinogen 1 (200 µl) was crosslinked under the standard buffer conditions by adding 0.4 mM DTT (panel A) or 0.05 mM DTT (panel B) and 50 µl of purified fXIII diluted to 105 Loewy u/ml or by adding 50 µl of pooled normal human plasma containing 105 u/ml fXIII. Fibrinogen 1 concentrations in the plasma-containing samples were adjusted so that the final fibrinogen concentration was 3 mg/ml. The reactions were incubated at room temperature for the indicated times, terminated by adding acetic acid, and the fibrinogen remaining in solution was quantified spectrophotometrically. (■) plasma factor XIII; (●) plasma. Mean of 3 independent experiments

Discussion

These present studies show that fibrinogen can be crosslinked and rendered insoluble during a Ca^{2+} dependent, thiol-enhanced reaction catalyzed by plasma factor XIII. In our hands, an easily appreciable level of crosslinking can be achieved at Ca^{2+} at concentrations above 50 µM, and that the effect is augmented at DTT concentrations above 2.5 µM. The results show that: 1, cellular and plasma factor XIII both

crosslink fibrinogen; 2, fibrin is crosslinked faster than fibrinogen; 3, fibrinogen 2, the carrier of plasma fXIII, is crosslinked more slowly and to a lesser extent than fibrinogen 1; 4, the fXIII purification process itself does not account for the observed crosslinking activity of fXIII, since plasma could more than adequately replace purified fXIII as a source of activity; 5, when fibrinogen or fibrin is crosslinked by plasma fXIII there is always an initial lag in the development of the maximal crosslinking rate; 6, fXIII crosslinking activity is specific for fibrinogen (or fibrin) as substrate(s) inasmuch as only very low transglutaminase activity was detected when cadaverine and N,N'-dimethylcasein were employed as substrates in the reaction. These data suggest that during the lag phase of the reaction fibrinogen substrate molecules bind at or near the active site of fXIII causing a conformational change that renders the "zymogen" catalytically active and that may also lead to dissociation of A and B subunits. Fibrinogen 2 slows the rate of crosslinking by binding the B subunits of the zymogen and restraining the diffusion of the catalytic A subunits.

Polgar et al. (68) reported that "platelet" (cellular) fXIII (A_2) could be activated by high concentrations of NaCl or KCl and that activation could be prevented by B subunits. Muszbek et al. (69, 70) have also shown that "platelet" fXIII could be activated intracellularly in a thrombin-independent manner. All of these activation phenomena required Ca^{2+}, occurred after secretion and platelet aggregation were completed, and resulted in the crosslinking of cellular proteins. In the latter two studies (69 ,70) the authors postulated that the activation process might require the interaction of "platelet" fXIII with some other cellular protein. The present results suggest the platelet component that could serve as substrate for uncleaved fXIII, may be fibrinogen itself. A conformational change in factor XIII induced by fibrin(ogen) in the presence of Ca^{2+} may be more important in the non-catalytic activation of fXIII than is removal of the activation peptide itself, since X-ray crystallographic studies on thrombin-cleaved recombinant cellular fXIII (A_2) demonstrated no large conformational changes in the molecule (71).

The pathophysiological role played by unactivated factor XIII in the circulation is unclear, but several considerations suggest that it is a readily available source of fibrin crosslinking activity during early phases of fibrin clot formation. Plasma factor XIII is cleaved relatively slowly by thrombin compared to thrombin cleavage of fibrinogen (47, 72), and thus crosslinking activity due to fXIIIa formation would be minimally available during early phases of thrombin-mediated clot formation. Moreover, since fibrin is a much better substrate for crosslinking than is fibrinogen (34, 35, this study), the crosslinking potential of clot-incorporated fXIII would be selective for fibrin over fibrinogen. Recent studies of Ancrod infusions in humans (73) strongly supports the idea that plasma fXIII is a potent source of crosslinking activity. Ancrod is a purified snake venom enzyme from the Malayan pit viper *Calloselasma rhodostoma* that clots fibrinogen by selectively cleaving fibrinopeptide A. Unlike thrombin, it has no capacity to cleave factor XIII (74). In their study, Dempfle et al. (73) measured the effects of Ancrod administration to normal volunteers, and early in the course of the infusion they found soluble crosslinked fibrin complexes, followed somewhat later by the appearance of a crosslinked fibrin degradation product, D dimer. Since there was no evidence that thrombin generation had occurred, these crosslinked products must have arisen through the constitutive crosslinking activity of factor XIII acting on Ancrod-induced fibrin.

There are several observations that probably reveal the footprint of fXIII activity in plasma. Normal plasma fibrinogen contains readily measurable amounts of intermolecularly crosslinked γ dimers and intramolecularly crosslinked Aα-γ heterodimers (75). The relative contribution by fXIII versus a tissue transglutaminase in forming these crosslinked fibrinogens cannot be distinguished, but nevertheless raises suspicion that fXIII is involved in their formation. α_2-antiplasmin is another possible substrate for the intrinsic activity of circulating fXIII. It has been known for some time that there is a potent plasmin inhibitory activity bound to fibrinogen (76). Although the specific entity in fibrinogen accounting for that activity was not identified, we infer from more recent studies (see below) that the inhibitory activity was attributable to the presence of α_2-antiplasmin covalently crosslinked to fibrinogen. Recently, Siebenlist et al. (77) demonstrated the presence of covalently bound α_2-antiplasmin in normal fibrinogen as well as in a dysfibrinogenemia known as fibrinogen Cedar Rapids (γR275C). We speculate that the α_2-antiplasmin had become incorporated into fibrinogen through the action of fXIII, in a manner that is entirely analogous to the crosslinking reaction that was demonstrated between fibrinogen and fXIIIa by Ichinose and Aoki (78). Further, the constitutive crosslinking activity of fXIII might conceivably also account for some or all of the crosslinked fibrinogen products that are found in basement membranes (79) and in atherosclerotic plaques (80). In addition, the intrinsic activity of fXIII could measurably contribute to formation of the crosslinked fibrin(ogen) γ chains that occur in pathological "cryofibrinogen" complexes (81, 82).

Familial Mediterranean Fever (FMF) is an hereditary disease in which cryofibrinogenemia is known to occur (83, 84) and is associated with increased fibrin(ogen) crosslinking (84). The gene responsible for causing the disease has been cloned (85), but the pathogenesis of the disorder remains uncertain. FMF is characterized by intermittent febrile episodes, abdominal pain, pleurisy, and arthritis (86). A substantial proportion of individuals with untreated disease eventually develop amyloidosis and renal failure (83). Although cryofibrinogenemia occurs commonly in FMF subjects, and is usually regarded as a marker for a hypercoagulable or thrombotic state (81, 82), FMF patients do not manifest a thrombophilic phenotype (83). The cryofibrinogen precipitates found in FMF contain high levels of crosslinked fibrinogen γ and (A) α chains (84), and it seems possible that some of the clinical manifestations of FMF could be related to enhanced fXIII activity leading to formation of increased levels of circulating crosslinked fibrinogen.

Moaddel et al. (87) recently concluded from the results of sedimentation equilibrium experiments on plasma fXIII-fibrinogen mixtures containing 1 mM $CaCl_2$, that high affinity non-covalent complexes had formed between fXIII and either fibrinogen 1 (γ_A, γ_A) or fibrinogen 2 (γ_A, γ'). In the case of fibrinogen 1, the apparent association constant (K_a) was 1.3×10^8, and was calcium-dependent because no complexes formed in the presence of EDTA. The apparent K_a for the fibrinogen 2 (γ_A, γ')-factor XIII "complex" was nearly thirty-fold higher, 3.6×10^9. These K_a values seem quite high in their own right for a presumed non-covalent molecular interaction, especially considering an earlier report that only γ'-containing fibrinogen 2 had any demonstrable binding affinity for factor XIII (9). Their interpretation that non-covalent complexes had formed depended upon the assumption that unactivated factor XIII could not promote crosslinking of fibrinogen in the presence of Ca^{2+}, which we show here to be incorrect. Thus, conclusions concerning non-covalent complex formation between fXIII and fibrinogen will have to be validated by a different set of experiments, since it seems certain that factor XIII-crosslinked fibrinogen molecules had been produced during the course of their sedimentation equilibrium experiments.

Under normal conditions only small amounts of crosslinked fibrinogen molecules and other proteins, such as α_2-antiplasmin, are found in

plasma. The typical calcium concentrations used in these experiments are comparable to those that exist in plasma. Moreover, there are thiols in the circulation such as glutathione, that can serve to augment the baseline enzymatic potential of fXIII. Recent reports indicate that thiol levels in plasma can be greater than 300 μM (88). It therefore seems very likely that plasma contains "inhibitory elements" that suppress or modulate the intrinsic fXIII activity. One such factor would be fibrinogen 2 itself, given that the γ' chain-containing, fXIII-binding form of fibrinogen becomes crosslinked by fXIII at a much lower rate than fibrinogen 1. The high levels of B subunits that are present in plasma might provide a second suppressive element, since B subunits are known to prevent thrombin-independent activation of "platelet" factor XIII (68) and their presence results in a lag in the onset of crosslinking by plasma fXIII. Another important down-regulating effect would be the fact that fibrin is a better substrate for fXIII crosslinking than is fibrinogen (34, 35, this study) and therefore the rate at which crosslinking takes place in the normal fibrinogen-containing plasma environment is typically much lower than it would be if fibrin were present.

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