

Contribution of Fibrin Stabilization to Clot Strength

SUPPLEMENTATION OF FACTOR XIII-DEFICIENT PLASMA WITH THE PURIFIED ZYMOGEN

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ABSTRACT The contribution of fibrin stabilization to clot strength, measured as the static elastic modulus, was evaluated in human plasma by two independent procedures. In the first approach, amine inhibitors of fibrin stabilization were examined for their effects on the rigidity of normal plasma clots. It is a unique property of these inhibitors that they do not interfere with the reversible aggregation of fibrin molecules, i.e., do not delay clotting time, but selectively prevent only the formation of γ -glutamyl- ϵ -lysine protein-to-protein linkages. Though the compounds tested were of different chemical structures and potencies, a fivefold reduction in clot strength was obtained in each instance. This value of 20% of normal seems to correspond to the rigidity of the Factor XIII-deficient plasma clot because, as demonstrated by the second approach, when a plasma specimen that genetically lacked the fibrin stabilizing factor was supplemented by the addition of measured amounts of the purified zymogen, a fivefold increase in clot strength could be achieved. The described procedure of evaluating Factor XIII in terms of correcting the elastic modulus of a deficient plasma clot is considered an important assay for the functional competence of purified preparations of the zymogen for the purpose of therapeutic application.

INTRODUCTION

The genetic lack of Factor XIII as well as all the other molecular disorders of fibrin stabilization due to the appearance of circulating inhibitors against either

zymogen activation or the functioning of the active enzyme on fibrin, can give rise to life-threatening hemorrhages (1). Nevertheless, clinically measured clotting times are invariably normal in these patients. The key problem, therefore, seems to be some physical difference between the normal clot structure that contains γ -glutamyl- ϵ -lysine linkages, and the structure that does not. Work with purified clotting components already has led to the conclusion that the introduction of even a few isopeptide side chain bridges caused a marked increase in the rigidity of the clot network (2-6). Alternately, the addition of primary amines such as glycine ethyl ester, histamine, or hydroxylamine, which specifically blocked the formation of inter-fibrin linkages (7), brought about a reduction of clot elasticity (8, 9).

We now report the results of a detailed study, using a Couette elastometer, for measuring static elastic moduli (or rigidities) of clots formed in normal and in Factor XIII-deficient human plasmas. In addition to examining inhibitory potencies of various primary amines on normal plasma, we have titrated the corrective effect of measured doses of purified Factor XIII on a plasma deficient in this zymogen.

METHODS

Blood was obtained from healthy subjects in one-tenth volume of 3.8% trisodium citrate, and platelet-poor plasma was prepared by centrifugation at 4,000 *g* for 20 min. Citrated, Factor XIII-deficient human plasma (lot GK 1302-930) was obtained from George King Bio-medical, Inc. (Overland Park, KS). Human α -thrombin was a gift from Dr. J. W. Fenton II, of the Department of Health, State of New York, Albany, NY. Plasma and thrombin samples were stored in the frozen state. The fibrin-stabilizing factor zymogen (Factor XIII) was isolated from outdated human plasma by procedures previously described (10, 11) and was stored at 4°C.

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The functional molarity of the product was assayed by titration with iodoacetamide (11, 12). The concentration of fibrinogen in the plasma samples was determined by the procedure of Astrup et al. (13).

Measurements of clot elasticities were carried out at 37°C in a Couette-type of apparatus recently described (14, 15). Clots were formed in a 1-mm gap between a sample cup and a concentrically suspended disc. When stressed by means of a magnetic coil device (shear stress), the disc rotates to a new equilibrium position (shear strain). With the latter parameter not exceeding 0.05, a linear relationship between shear stress and shear strain is obtained, and under a constant shear stress (1 dyn/cm²), the shear strain is inversely proportional to clot strength. The ratio of shear stress to shear strain is the static elastic modulus: G ,¹ expressed in dyn/cm². To obtain a kinetic profile of the change of G value in the clot, measurements were made at frequent time intervals after the first sign of clotting. Typically, 1 ml of citrated plasma sample was mixed with 0.1 ml of 1 M HEPES buffer of pH 7.6, 0.1 ml of Trasylol (Mobay Chemical Corp., FBA Pharmaceuticals, New York; 100 kallikrein U/ml of 0.15 M NaCl) and 0.6 ml of 0.15 M NaCl in which, as required, primary amines or purified Factor XIII were included as additives. Clotting was initiated by the addition of 0.2 ml of 0.15 M NaCl, containing 1 NIH U of thrombin and 0.18 M CaCl₂.

Some clots were preserved for electrophoretic studies (16) by freezing them at the conclusion of elastometric measurements. Upon thawing, the clots were washed for a total period of 24 h with four changes of 0.15 M NaCl (10 ml each time), containing 1 mM EDTA and 50 mM hydroxylamine at pH 7.5, and were rinsed with distilled water. Aliquot pieces of ~0.5 mg were removed, incubated for 15 h at 37°C in a solution of 1% sodium dodecylsulfate, 4.5 M urea, and 1% β -mercaptoethanol, and boiled for 5 min. The concentration of β -mercaptoethanol was raised again by adding 1% of the reducing agent before electrophoresis on 5% polyacrylamide gels (100 mA for 12 h; 0.1 M phosphate buffer, pH 7.1, containing 0.1% sodium dodecylsulfate).

RESULTS

The effect of amine inhibitors on clot strength in normal plasma. The effect of the smallest inhibitor, hydroxylamine (17) was examined in normal plasma in a systematic manner. As shown in Fig. 1, soon after the onset of clotting (~1 min), first there was an initial rapid increase, then a much slower increase in G value. Even though no final value of G was ever reached within the experimental period of 60–120 min, it was clear that the addition of increasing concentrations of hydroxylamine to the clotting mixture caused a substantial decrease of elastic modulus. When the G values for 60 min were plotted against the initial concentration of hydroxylamine, the results presented in Fig. 2 were obtained. To guard against the possibility that the reduction of G values could in any way be due to the degradation of the fibrin network by plasmin, parallel experiments were performed in the presence of

¹ Abbreviation used in this paper: G , ratio of shear stress to shear strain, static elastic modulus.

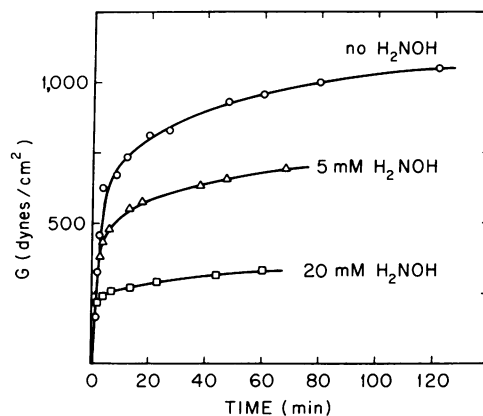


FIGURE 1 Increase of elastic modulus during clotting in normal plasma, with and without hydroxylamine added. Fibrinogen content was 2.85 mg/ml.

Trasylol, but the data were indistinguishable from those without plasmin inhibitor. A noteworthy aspect of the experiments with hydroxylamine was the finding that the limiting value to which the rigidity of plasma clots could be reduced was ~20% of normal. The reduced sodium dodecylsulfate electrophoretic profile of the clot formed in the presence of 30 mM hydroxylamine was essentially that of an unligated structure (see insert in Fig. 2) and, by contrast to the clot obtained in the absence of hydroxylamine, no α_n polymer was detectable. Furthermore, the intensities of bands corresponding to the α -, β -, and γ -chains

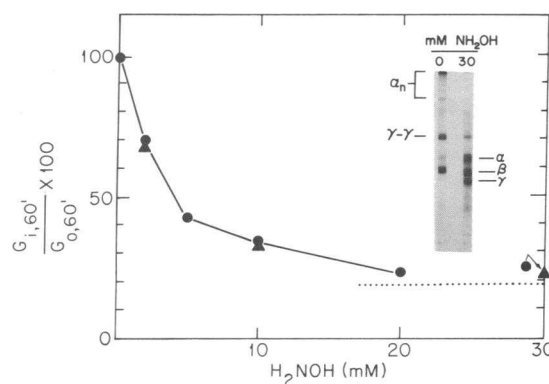


FIGURE 2 The effect of hydroxylamine on the elasticity of normal plasma clot in the presence (●) and absence (▲) of Trasylol. The ordinate denotes the percentage of the elastic modulus at 60 min with hydroxylamine as inhibitor ($G_{60,60'}$) in relation to no inhibitor added ($G_{0,60'}$). The insert shows the sodium dodecylsulfate electrophoretic profiles, after reduction, of Trasylol-containing clots formed in the absence of hydroxylamine (left-hand gel), and in the presence of 30 mM of this amine (right-hand gel).

of fibrin were all very strong, and only minimal γ -chain dimerization (<2%) was visible.

It is known that primary amines, depending on their structure and pK_a , show different potencies for inhibiting fibrin stabilization when examined by the criterion of clot solubility in 1% monochloroacetic acid (7). Of the compounds available, we have chosen representative small amines (hydroxylamine and aminoacetonitrile), some large monosubstituted cadaverines with various apolar (2,4-dinitrophenyl, mesitylenesulfonyl, and dansyl) residues and dansylthiacadaverine (18).

When the elastic moduli of clots formed in normal plasma were examined as a function of the concentration of added amines (Fig. 3), it became apparent that, regardless of the chemical nature and potencies of the inhibitors, the lowest limiting value for G in plasma with thrombin and Ca^{2+} , was $\sim 20\%$ of normal.

The concentration needed to bring about a half-maximal reduction of the elastic modulus (i.e., to 60%) varied between inhibitors, and the required concentrations, as read off from the abscissa in Fig. 3, showed an approximately ninefold spread between dansylcadaverine and hydroxylamine. This, however, does not provide a proper comparison of relative inhibitory potencies for the compounds tested because of the wide differences of the pK_a of the primary ammonium ions. If, instead of the total added concentration of a given amine (i.e., the sum of amine and ammonium ion forms), the effective concentration of the deprotonated nucleophile that is considered to be the inhibitory species (7, 18) was calculated for the pH of the experiment (pH 7.6), a completely different appraisal emerged. The data, together with the chemical structures and pK_a of the inhibitors, are given in Table I

and, for the series examined, the relative order of inhibitory potencies (in square brackets) was dansylcadaverine \approx mesitylenesulfonylcadaverine, [9,000], dansylthiacadaverine, [4,514], 2,4-dinitrophenylcadaverine, [1,514], aminoacetonitrile, [2], and hydroxylamine [1].

The clot strength of Factor XIII-deficient plasma with and without supplementation by purified Factor XIII. Considering the finding mentioned above that all the amine inhibitors of fibrin stabilization reduced the elastic modulus of normal plasma clot to a common limiting value, this basic clot strength of 20% of normal was thought to correspond to what might be found with a Factor XIII-deficient plasma. Indeed, as shown in Fig. 4, this supposition was shown to be correct when clots produced by the addition of Ca^{2+} and thrombin to a deficient plasma sample were examined. To prove that the very low G value of ~ 100 dyn/cm² was entirely due to the absence of the zymogen from the deficient plasma and not to some unknown solute, measurements were made in the presence of incremental doses of purified human Factor XIII. Added concentrations of purified Factor XIII supplements, as determined by active site titration (11, 12), ranged from 2×10^{-10} to 10^{-7} M. By adding 5×10^{-8} M of Factor XIII (Δ in Fig. 4), which is in the normal range of concentration for the zymogen in plasma, the highest possible value in G was reached in ~ 30 min, resembling the curve for the elastic modulus of a clot formed in normal plasma. Significantly, the plateau in G was about five times greater (\circ and Δ in Fig. 4) than that without supplementation by the zymogen. Thus, the latter (\blacktriangledown in Fig. 4) clearly represents the basic elastic modulus of a clot structure formed in plasma in the presence of Ca^{2+} but without any pos-

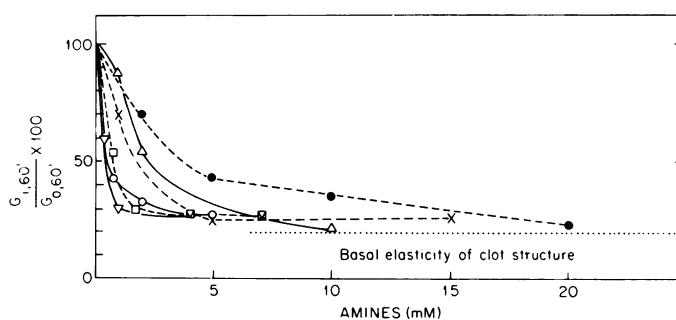


FIGURE 3 Relative efficiency of inhibitors of fibrin stabilization in reducing the elasticity of normal plasma clots. Ordinate is same as in Fig. 2. Dansylcadaverine (∇), mesitylenesulfonylcadaverine (\circ), dansylthiacadaverine (\square), aminoacetonitrile (\times), 2,4-dinitrophenylcadaverine (Δ), and hydroxylamine (\bullet) concentrations are shown on the abscissa. Chemical structures and pK_a for the primary ammonium functions of these compounds are given in Table I. The horizontal dotted line, referred to as the basal elasticity of clot structure, corresponds to an elastic modulus of 20% of normal.

TABLE I
Potencies of Inhibitors of Fibrin Stabilization for Reducing the Elastic Moduli of Clots Formed in Normal Plasma

Name	Compound Structure	pK _a of primary ammonium ion	Inhibitory potency*		
			From Fig. 3	pK _a - corrected potency	Corrected relative potency
			$M^{-1} \times 10^{-3}$	$M^{-1} \times 10^{-3}$	
Hydroxylamine	H ₂ NOH	5.97†	0.37	0.36	(1)
Aminoacetonitrile	H ₂ NCHCN	5.5†	0.77	0.77	2
2,4-Dinitrophenylcadaverine	H ₂ N(CH ₂) ₅ NHC ₆ H ₃ (NO ₂) ₂	10.6§	0.56	560	1,514
Mesitylenesulfonylcadaverine	H ₂ N(CH ₂) ₅ NHSO ₂ C ₆ H ₂ (CH ₃) ₃	10.6§	3.33	3,330	9,000
Dansylcadaverine	H ₂ N(CH ₂) ₅ NHSO ₂ (C ₁₀ H ₄)N(CH ₃) ₂	10.6§	3.33	3,330	9,000
Dansylthiacadaverine	H ₂ N(CH ₂) ₂ S(CH ₂) ₂ NHSO ₂ (C ₁₀ H ₄)N(CH ₃) ₂	9.6§	1.67	1,670	4,514

* The inhibitory potency is defined as the reciprocal concentration of inhibitor required to bring about half-maximal lowering of elastic modulus; here it is based on the data of Fig. 3.

† See reference 19.

§ See reference 18.

sibility of fibrin stabilization. If clotting was induced by thrombin alone, with the omission of Ca²⁺, a further twofold reduction in G value ensued (--- in Fig. 4).

The two inserts in Fig. 4 show the reduced sodium dodecylsulfate electrophoretic profiles for the 65- and

60-min clots, corresponding respectively to the experiments without Factor XIII supplementation (▼; lower right-hand insert) and with 10⁻⁷ M of the purified zymogen (○; upper left-hand insert). In the patient's plasma clot, practically no ligation was found, but upon supplementation with Factor XIII, there was a complete disappearance of monomeric γ- and α-chains, with concomitant appearance of γ-γ dimers and various α-polymers.

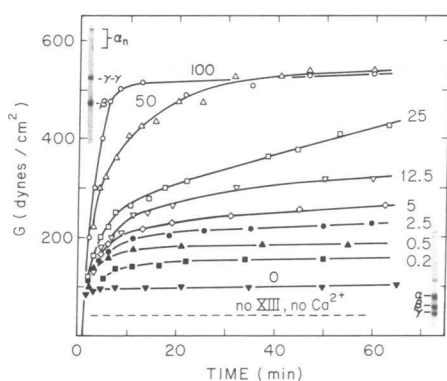


FIGURE 4 Increase in the elastic modulus of clot in a Factor XIII-deficient plasma, as a function of supplementing with measured doses of the purified Factor XIII zymogen. Added concentrations of the latter, based on active site titrations with iodoacetamide (11, 12), ranged from 0.2×10^{-9} M to 100×10^{-9} M and are indicated on the graph ($\times 10^9$) for each experiment. The line marked "0" (▼) at ~ 100 dyn/cm² represents the strength of the clot formed in Factor XIII-deficient plasma, with thrombin and Ca²⁺ added but without supplementation with purified Factor XIII. The broken line at ~ 40 dyn/cm² is the elasticity of the clot obtained in Factor XIII-deficient plasma just with the addition of thrombin. Fibrinogen content was 1.88 mg/ml. The inserts show the electrophoretic profiles for reduced clots in sodium dodecylsulfate for the 65-min sample from the deficient plasma (▼; lower right) and for the 60-min sample from the 10⁻⁷ M Factor XIII-supplemented material, (○; upper left).

DISCUSSION

The clotting of fibrinogen in plasma involves a complex series of events and interactions; the outline is given in Fig. 5.

The clotting time measured in the clinical laboratory represents the aggregation of fibrin units: n fibrin \rightleftharpoons (fibrin)_n, and the gel point corresponds to the situation when the fibrinopeptide A moieties have been cleaved by thrombin from about one-fourth of the fibrinogen molecules in solution. However, completion of clotting requires not only the release of all the residual fibrinopeptide A's and B's, but involves also the activation of fibrin stabilizing factor (Factor XIII) and the subsequent enzymatic fusion (ligation, cross-linking) of the fibrin molecules by γ-glutamyl-ε-lysine linkages. When there is an abnormality in the operation of the fibrin stabilizing system, be it due to the genetic lack of the zymogen itself or to the presence of an acquired inhibitor directed either against the activation of this zymogen (marked by ⊙ in Fig. 5) or against the action of the Factor XIII_a enzyme on fibrin (marked by ⊖ and ⊕ in Fig. 5), severe hemorrhagic conditions may arise (1). Inasmuch as the clotting times in these quite

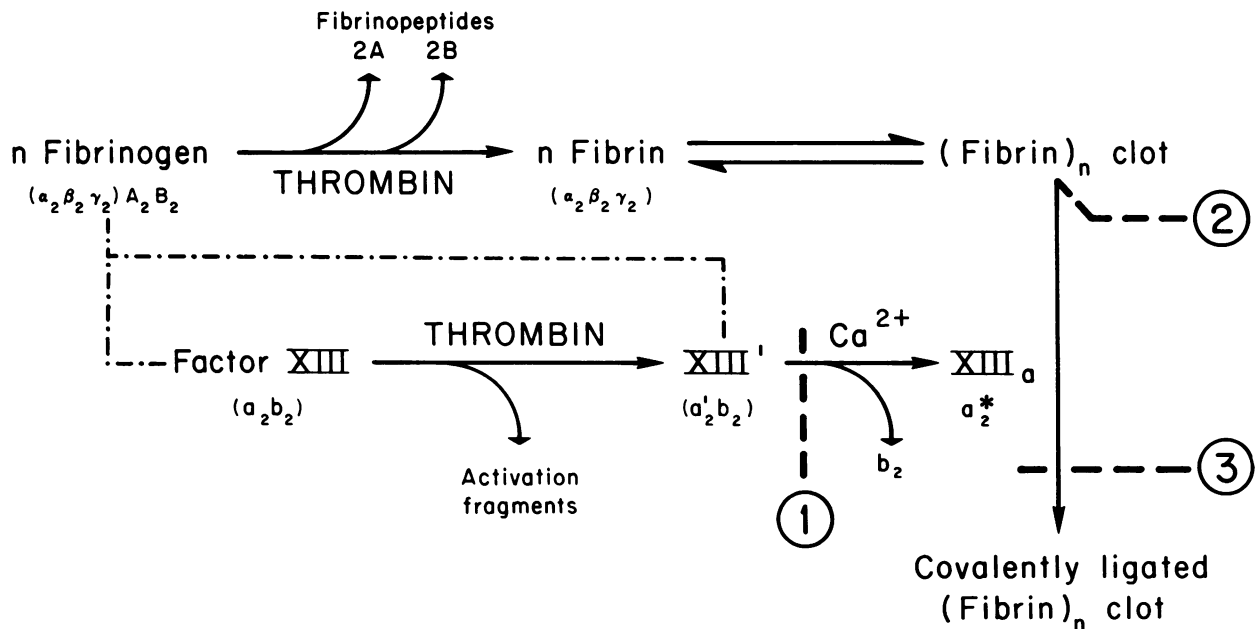


FIGURE 5 Outline of the clotting reaction in normal human plasma. Disorders of fibrin stabilization can be genetic, such as Factor XIII deficiency, or acquired due to the appearance of circulating inhibitors directed against one of the steps in the sequence marked with broken lines (---) as ①, ②, and ③. For a discussion of the various molecular diseases, consult reference 1. The dotted lines (- · - · -) refer to the regulation exerted by fibrinogen on the conversion of the Factor XIII zymogen, in regard to enhancing the rate of release of activation fragments by thrombin (26) and lowering of Ca^{2+} requirement (27).

different molecular disorders are normal, explanation for the bleeding that is usually caused by secondary oozing must be sought in the physical characteristics of the clot itself.

To evaluate the selective contribution to clot strength of the functioning of the Factor XIII system, we took advantage of the availability of specific inhibitors of fibrin stabilization (7) and measured their effects on the elastic modulus of the clot formed in normal plasma in the presence of Ca^{2+} . These inhibitors, which prevent the formation of γ -glutamyl- ϵ -lysine linkages by competing against the reactive lysine residues in the protein substrate, have long been known to augment the susceptibility of blood clots to lysis by plasmin (20). It has also been suggested that this was achieved by preventing the covalent addition of α_2 -plasmin inhibitor to fibrin by such linkages (21).

It is a significant finding that all the inhibitory compounds were able to reduce G values to about one-fifth of normal (Fig. 3), which can thus be regarded as the basic elasticity of the fibrin structure in the presence of Ca^{2+} but without stabilization. It is known that the amines act as inhibitors by virtue of becoming incorporated into the Factor XIII_a-reactive γ -glutamine sites and thereby prevent the formation of protein-to-protein γ -glutamyl- ϵ -lysine bridges (7, 17). Thus, with

the compounds used, modification of the γ -glutamine sites in fibrin (F) would vary from the relatively minor alteration of $\text{F-CONH}_2 \rightarrow \text{F-CONHOH}$ for hydroxylamine, to $\text{F-CONH}_2 \rightarrow \text{F-CONHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHSO}_2$ ($\text{C}_{10}\text{H}_{14}$) $\text{N}(\text{CH}_3)_2$ for dansylcadaverine. Yet even the latter, which involves the insertion of a bulky apolar residue and a long sidearm into the area of contact domains of fibrin, does not seem to cause a large enough perturbation in the clot network to reduce the elastic modulus below 20%.²

This 20% of normal seems to be the basic elasticity also by the criterion of analyzing the clot in Factor XIII-deficient plasma (Fig. 4). When the deficient plasma was supplemented with purified human zymogen, a fivefold correction of elastic modulus was the highest value achievable.³ It might be mentioned

² Measuring the potencies of inhibitors of fibrin stabilization by the elastometric procedure is an important tool for evaluating the action of such compounds on clot formation in normal plasma.

³ The difference in the actual G values at 60 min between the fully corrected patient sample (○ in Fig. 4) and the uninhibited normal (○ in Fig. 1) may be attributed to the different fibrinogen contents of the two plasma specimens (1.88 mg/ml in Fig. 4 and 2.85 mg/ml in Fig. 1), because fibrinogen concentration is known to have an appreciable influence on G values (14).

that by using purified clotting components, the addition of Factor XIII to fibrinogen, thrombin, and Ca^{2+} produced varying increments in G value (3, 4, 22).

The evaluation of measured doses of Factor XIII in terms of correcting the elastic modulus of a Factor XIII-deficient plasma clot is of considerable practical significance, because the procedure as outlined in Fig. 4 offers the best means for testing the functional competence of the purified zymogen. Other quantitative methodologies, based on measuring the concentration of active sites by reaction with iodoacetamide (11, 12) or the velocity of incorporation of amines into casein derivatives (23–25) are further removed from the real physiological function of this protein to be considered completely reliable indices for standardizing the zymogen for purposes of therapeutic infusion into patients with Factor XIII-deficiency. The question as to how a purified product contributes to normalization of the physical impairment of the deficient clot structure might be better answered by the type of experiment shown in Fig. 4.

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