Increased Resistance to Plasmic Degradation of Fibrin With Highly Crosslinked \(\alpha\)-Polymer Chains Formed at High Factor XIII Concentrations

By Charles W. Francis and Victor J. Marder

We have previously demonstrated that increasing factor XIII concentrations above that present in plasma (1 U/mL) results in the formation of very high molecular weight \(\alpha\) fibrin polymers.5 In this report, we have examined the effect of such crosslinking on plasmic susceptibility of fibrin prepared from purified fibrinogen and from plasma in the presence of factor XIII concentrations between 0 and 10 U/mL. The crosslinking achieved with purified fibrinogen at 1 U/mL factor XIII increased resistance to plasmic degradation by 32% as measured in a radiolabeled clot lysis system. However, further increases in plasmic resistance occurred at factor XIII concentrations of 2 and 10 U/mL, the latter decreasing the lysis rate to 45% of that which occurred in the absence of factor XIII. To achieve the same rate of clot lysis with fibrin formed using 10 U/mL rather than 1 U/mL of factor XIII, an increase in plasmic concentration of up to 4.2-fold was required. Similar results were obtained using clots prepared from plasma in the presence of factor XIII concentrations greater than 1 U/mL. Since the \(\alpha\)2-plasmin inhibitor content was the same for fibrin at 1 or 10 U/mL factor XIII, the increasing plasmic resistance could not be attributed to increased binding of the inhibitor. We conclude that fibrin prepared in the presence of factor XIII at concentrations exceeding that in plasma shows increased resistance to plasmic degradation, which is likely explained by the formation of very high molecular weight \(\alpha\) polymer chains.

\(\text{FOLLOWING ACTIVATION}\) by thrombin, factor XIII, stabilizes the fibrin gel through formation of intermolecular epsilon-(\(\gamma\)-glutamyl) lysine covalent cross-links.3 Rapid crosslinking occurs between pairs of adjacent \(\gamma\) chains,2 and the \(\alpha\) chains are crosslinked more slowly, with each \(\alpha\) chain crosslinked to up to two others, forming polymers.3,4 We have recently shown5 that fibrin prepared from normal plasma, which contains 1 U/mL factor XIII, shows incomplete \(\alpha\) chain crosslinking with residual \(\alpha\)-monomer and formation of only small \(\alpha\)-polymer chains. However, a much greater extent of \(\alpha\) chain crosslinking could be induced with higher factor XIII concentrations as supplied by purified factor XIII or by platelets, which contain nearly 50% of blood factor XIII.6,7

Crosslinking affects several properties of fibrin gels, rendering them insoluble in weak acid or urea and increasing their rigidity and elasticity.9,10 Crosslinking of fibronectin to fibrin11 affects its mechanical properties12,13 and also increases the migration of cells into the gel in vitro.14 Many studies have considered the influence of crosslinking on susceptibility to plasmic degradation, with most15-20 but not all,21-23 studies finding that fibrin crosslinking confers greater resistance to plasmic degradation. Interpretation of these studies is difficult in view of recent evidence demonstrating that crosslinking has effects on fibrin that influence fibrinolytic susceptibility independent of fibrin chain crosslinking. Sakata and Aoki23,24 showed that \(\alpha\) plasmin inhibitor crosslinks to fibrin \(\alpha\) chains, increasing plasmic resistance. Also, Sakata et al25 showed decreased binding of plasminogen to crosslinked compared with noncrosslinked fibrin, complicating interpretation of studies using plasminogen activators. Finally, prior studies did not consider the effect of factor XIII concentrations greater than that in plasma and did not fully characterize the extent of \(\alpha\) chain crosslinking with methods capable of distinguishing \(\alpha\) polymers of large size.2 In the present study, we have evaluated the effect of \(\alpha\) chain crosslinking on susceptibility of fibrin to plasmic lysis, using plasmin rather than plasminogen activators, controlling the incorporation of \(\alpha\) plasmin inhibitor, and using factor XIII concentrations up to 10 U/mL with characterization of the extent of \(\alpha\) chain polymerization and characterization of the extent of \(\alpha\) chain polymerization and the size of \(\alpha\) polymer chains.

METHODS

Proteins. Fibrinogen (grade L) was purchased from Helena Laboratories (Beaumont, TX), and factor XIII deficient fibrinogen was prepared as described.6 Fibrinogen was \(\text{\textsuperscript{125}}\text{I}\)-labeled using the iodogen technique27 to a specific activity of 5 Ci/mmol, and the bound and unbound \(\text{\textsuperscript{125}}\text{I}\) were separated by chromatography on Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ). The clottability of labeled fibrinogen was 93%, and the electrophoretic mobility on sodium dodecyl sulfate (SDS) 7% polyacrylamide gel electrophoresis (PAGE) was the same as unlabeled fibrinogen. Human thrombin with a specific activity of 1,000 NIH U/mg was purchased from Calbiochem (La Jolla, CA). Purified factor XIII concentrate (Fibrogammin) prepared from human placenta was kindly provided by Behringwerke/Hoechst-Roussel (Somerville, NJ). Factor XIII was assayed by dansyl cadaverine incorporation into casein28 and by Laurell electroimmunossay using an antibody directed against the factor XIII \(\alpha\) chain (Cappell Laboratories, Cochranville, PA) and expressed in relation to pooled normal plasma defined as 1 U/mL. Plasmin with an activity of 10.2 Committee on Thrombolytic Agents U/mL was kindly provided by Dr David Aronson (Bureau of Biologies Standards, Bethesda, MD).

Plasma. After obtaining informed consent, blood was obtained by venipuncture from normals and collected into citrate (.4% final

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concentration). Since platelets contain factor XIII, care was taken to prepare plasma completely depleted of platelets. Blood was initially centrifuged at 2,300 g for 15 minutes to prepare plasma containing 20,000 to 30,000 platelets per microliter, and this plasma was again centrifuged at 15,000 g for ten minutes to obtain plasma containing <3,000 platelets per microliter as determined by phase contrast microscopy. Pooled normal plasma was prepared from at least ten donors. Factor XIII deficient plasma was obtained from George King Biochemicals (Overland Park, KA).

Clot preparation. Fibrinogen was diluted to 5 mg/mL with .15 mol/L sodium chloride, .05 mol/L Tris-hydrochloric acid buffer, pH 7.4, factor XIII added to the desired concentration, and the solution clotted by the addition of calcium chloride (50 mmol/L, final concentration) and thrombin (1 U/mL, final concentration) with no sulphydryl containing reagent. Clots from 0.3 mL were prepared in 1.5 mL conical plastic tubes, incubated at 25°C for 18 hours, and then centrifuged at 12,000 g for two minutes. The supernatant was removed and the clots washed twice with 5.0 mL of .15 mol/L sodium chloride, .05 mol/L Tris-hydrochloric acid buffer, pH 7.4. Plasma clots were prepared in the same way.

Coagulation assay. A rabbit antiserum to human a2 plasmin inhibitor (Behring Diagnostics, La Jolla, CA) was used with pooled normal plasma as a standard. Incorporation into plasma clots was determined by subtracting inhibitor content of serum and clot washes from that in the plasma before clotting.

Clot lysis. Washed clots were suspended in .5 mL of .15 mol/L sodium chloride, .05 mol/L Tris-hydrochloric acid buffer, pH 7.4, and plasmin added to the desired concentration. The tubes were rotated 40 times per minute at room temperature. Lysis was routinely monitored by following the increase in optical density at 280 nm and calculation of soluble protein using an extinction coefficient of 15.1. The results of the two methods were in close agreement.

Electrophoresis. Electrophoresis in SDS polyacrylamide gels or in SDS 2% agarose gels was performed as described. Fibrin was dissolved for electrophoresis with disulfide bond reduction by incubating in SDS containing diluent at 60°C for two to 16 hours. For agarose gels the diluent was .01 mol/L phosphate buffer, pH 7, containing 1.7% (wt/vol) SDS and 5% (vol/vol) β-mercaptoethanol; and for polyacrylamide gels .1 mol/L phosphate buffer, pH 7, containing 10 mol/L urea, 5% (wt/vol) SDS and 5% (vol/vol) β-mercaptoethanol. Gels were stained for protein with Coomassie Brilliant Blue (Sigma Chemicals, St Louis), or autoradiograms prepared from dried gels using Kodak X-omat (XAR-a) film days.

RESULTS

Fibrin was prepared from fibrinogen at increasing factor XIII concentrations, and the extent of α chain crosslinking assessed by electrophoresis in 5% SDS-polyacrylamide gels to determine the amount of residual α-monomer (Fig 1, left) and in 2% agarose gels to examine the size range of α-polymers (Fig 1, right). Factor XIII-deficient fibrinogen showed no evidence of γ dimer or α-polymer formation, while γ dimer formation was complete at concentrations of .5 U/mL and above. However, higher factor XIII concentrations were required for α chain crosslinking. At .5 U/mL the proportion of α-monomer was only slightly less than in the factor XIII-deficient fibrinogen, and only lower molecular weight α-polymer chains were noted, migrating only slightly slower than the γγ band on the 2% agarose gel. At 1 and 2 U/mL factor XIII there was progressively less residual α-monomer, and at 10 U/mL no α monomer was seen, while the proportion of higher molecular weight α-polymers seen on the SDS 2% agarose gel increased progressively at 1, 2, and 10 U/mL. These higher molecular weight forms included an increasing amount of more slowly migrating α-polymer forms and also a portion that failed to enter the 2% gel.

Fibrin was prepared at factor XIII concentrations from 0 to 10 U/mL and digested with plasmin to examine the effect of increasing α chain polymerization on susceptibility to lysis (Fig 2). At all factor XIII concentrations, the rate of fibrin solubilization was linear, and it decreased progressively at higher factor XIII concentrations up to 10 U/mL. The lysis rate of fibrin prepared at 1 U/mL factor XIII was 1,790
Fig 2. Plasmic lysis of fibrin prepared at varying factor XIII concentrations. Fibrin was prepared from purified fibrinogen at varying factor XIII concentrations and the polypeptide chain composition determined as in Fig 1. Following harvesting and washing, the fibrin was suspended in buffer containing .025 U/mL plasmin, incubated at 25°C, and the rate of fibrin lysis determined from measurement of the solubilization of radiolabel. The factor XIII concentrations used are indicated (U/mL).

μg/min/U plasmin, 68% of the rate of noncrosslinked, factor XIII-deficient fibrin (Table 1). The lysis rate decreased further at 2 and 10 U/mL, at the latter concentration being 1,180 μg/mL/U plasmin or 45% of the rate of 0 U/mL.

To test reproducibility, fibrin was prepared on six separate occasions at 1 and 10 U/mL factor XIII, the extent of crosslinking assessed electrophoretically, and lysis rates determined (Fig 3). Despite some variability, the lysis rate of fibrin prepared at the higher factor XIII concentrations was consistently lower with the rate at 10 U/mL being 47% of the rate of factor XIII deficient fibrin, in agreement with the results in Table 1.

To determine if the difference in susceptibility to lysis was dependent on plasmin concentration, fibrin was prepared at 1 U/mL and 10 U/mL factor XIII and lysis rates determined over a range of plasmin concentrations (Fig 4). At plasmin concentrations between .0025 and .25 U/mL, there was a progressive increase in lysis rates. At all plasmin concentrations tested, the lysis rate was lower with fibrin prepared at 10 units compared with 1 U/mL factor XIII. To achieve 3% lysis at two hours under the conditions of the experiment a threefold increase in plasmin concentration was required with fibrin prepared at 10 U/mL as compared with 1 U/mL.

Table 1. Lysis Rates of Fibrin Prepared at Increasing Factor XIII Concentrations

<table>
<thead>
<tr>
<th>Factor XIII Concentration</th>
<th>Lysis Rate (μg/min/U Plasmin)</th>
<th>Relative Rate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.630</td>
<td>100%</td>
</tr>
<tr>
<td>.5</td>
<td>2.240</td>
<td>85%</td>
</tr>
<tr>
<td>1</td>
<td>1.790</td>
<td>68%</td>
</tr>
<tr>
<td>2</td>
<td>1.540</td>
<td>59%</td>
</tr>
<tr>
<td>10</td>
<td>1.180</td>
<td>45%</td>
</tr>
</tbody>
</table>

Data taken from Fig 2.

*Lysis rates as percent, based on 100% at 0 U/mL.

Fig 3. Rate of lysis of fibrin prepared at varying factor XIII concentrations. Data obtained from six separate experiments using fibrin prepared from factor XIII-depleted fibrinogen, and at 1 and 10 U/mL is combined. The plasmin concentration was .025 U/mL, and the rate of lysis determined by solubilization of radiolabel.

while 30% lysis required a 4.2-fold increase. Over the range studied, the average increase required at 10 U/mL factor XIII was approximately threefold.

Clots were also prepared from plasma instead of purified fibrinogen to determine if there was a similar dependence of lysis rate on factor XIII concentration and extent of α chain crosslinking. Pooled normal plasma containing no platelets and the same plasma supplemented with purified factor XIII to a concentration of 10 U/mL were clotted, and the extent of fibrin crosslinking confirmed by gel electrophoresis. At 1 and 10 U/mL factor XIII, the extent of α chain crosslinking and size of α-polymers formed were similar to that with purified fibrinogen (data not shown). Since plasma contains α2 plasmin inhibitor, which crosslinks to fibrin and inhibits fibrinolysis, the amount of α2 plasmin inhibitor binding was measured. Fibrin with 1 or 10 U/mL factor XIII had 45% and 40%, respectively, of bound α2-plasmin inhibitor bound (mean of two experiments), indicating that any difference in lysis rate could not be attributed to differences in inhibitor concentration or binding. To obtain a lysis rate for plasma
clots comparable with that with fibrin prepared from purified fibrinogen, which contained no α₂ plasmin inhibitor, a tenfold higher plasmin concentration was required. However, as shown in Fig 5, the rate of lysis remained strongly dependent on the factor XIII concentration used to prepare the fibrin with the rate at 10 U/mL factor XIII being 40% of the rate at 1 U/mL.

**DISCUSSION**

The data show that plasmic resistance of fibrin is higher in proportion to factor XIII concentration, and that this relationship pertains for concentrations that exceed that in plasma (1 U/mL). Thus, resistance at 2 U/mL was higher than that at 1 U/mL, and was correlated with more complete α chain crosslinking and the formation of the high molecular weight α-polymer chains (Fig 1). Comparison of lysis rates (Table 1) with gel patterns (Fig 1) suggests that the increased plasmic resistance parallels formation of the largest α-polymer forms, particularly those that fail to enter the 2% gel and are of a size comparable with the largest plasma von Willebrand factor multimers. Thus, although little α-monomer remained at 2 U/mL factor XIII (Fig 1, left), the greater plasmic resistance noted at 10 U/mL (Fig 2, Table 1) was associated with much larger α polymer chains that migrated more slowly or failed to enter the agarose gel (Fig 1, right).

Crosslinking of α₂ plasmin inhibitor to fibrin prepared from purified fibrinogen could not explain the increased resistance to lysis at higher factor XIII concentrations, since the fibrinogen preparation contained no detectable α₂ plasmin inhibitor. However, the tenfold higher plasmin concentration required to achieve lysis rates of fibrin prepared from plasma compared with that prepared from purified fibrinogen may be explained in part by binding of α₂ plasmin inhibitor in the plasma system. Since there was no increase in α₂ plasmin inhibitor binding to fibrin prepared from plasma at 10 U/mL factor XIII compared with 1 U/mL factor XIII, inhibitor binding could not explain the increased resistance to plasmic degradation at the higher factor XIII concentration (Fig 4). Also, the increased resistance could not be ascribed to decreased plasminogen binding to crosslinked fibrin, since plasmin and not plasminogen activator was used in lysis experiments. Our findings indicate that lysis susceptibility is significantly influenced by the greater α chain crosslinking that occurs at factor XIII concentrations above 1 U/mL, independent of α₂ plasmin inhibitor binding. However, the relative importance and possible additive or potentiating influences of fibrin chain crosslinking and α₂ plasmin inhibitor binding in plasma cannot be determined from the data.

Several prior reports have found an increased resistance to plasmic degradation of crosslinked compared with noncrosslinked fibrin, but the findings differ from those in the present report in several respects. First, the fibrin used in other studies was prepared from plasma at effective factor XIII concentrations of 1 U/mL or less by use of calcium chelation, factor XIII inhibitors, or mixtures of normal and factor XIII-deficient plasma to reduce factor XIII activity. These conditions would result in complete γ chain crosslinking, which requires only low factor XIII concentrations but would be insufficient for complete α chain crosslinking or formation of very large polymers, which occurs at factor XIII concentrations above that in normal plasma.

Both Schwartz et al. and Gaffney and Whitaker differentiated between α and γ chain crosslinking and concluded that resistance to lysis was associated with crosslinking of α chains. However, both studies prepared fibrin from plasma so that the increased resistance seen with factor XIII may have been due to incorporation of α₂ plasmin inhibitor, which was not measured. Both used plasminogen activators to lyse clots, rather than plasmin, so that decreased incorporation of plasminogen into crosslinked compared with noncrosslinked fibrin may also have contributed to the findings. Further, Gaffney and Whitaker used factor XIII concentrations of 1 U/mL or less and Schwartz et al. did not specify the factor XIII activity used so that the effects of factor XIII concentrations over 1 U/mL could not be determined. Finally, prior systems for assessing α polymer formation did not resolve α polymer chains of large size, and therefore the degree of α chain crosslinking and size of α polymer chains could not be determined.

The action of factor XIII has multiple effects on fibrin structure, which could influence susceptibility to plasmic lysis. In addition to decreasing plasminogen binding and increasing inhibitor crosslinking, factor XIII also crosslinks both fibronectin and von Willebrand factor to fibrin α chains. Both were present in low concentrations in the purified fibrinogen used, but incorporation of either protein has not been shown to influence fibrin susceptibility to enzymatic degradation. Factor XIII crosslinking also affects the physical properties of fibrin gels, increasing their rigidity and elasticity, changes which could influence gel permeability and affect the access of enzyme to the interior.
of the gel. However, the results presented in this report indicate that the increased size and complexity of highly crosslinked α polymer chains that are formed at high factor XIII concentrations contribute directly to increased plasmic resistance. The additional factor XIII may be provided by platelets, which accumulate at sites of vessel injury or thrombosis, suggesting that platelets contribute to the regulation of fibrinolysis by stabilizing the fibrin network through α chain crosslinking.

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