Increased plasma concentration of cross-linked fibrin polymers in acute myocardial infarction

CHARLES W. FRANCIS, M.D., D. GERARD CONNAUGHAN, M.D., WILLIAM L. SCOTT, M.D., AND VICTOR J. MARDER, M.D.

ABSTRACT Thrombin cleaves fibrinopeptides from fibrinogen, converting it to fibrin monomer, and activates factor XIII, which catalyzes the formation of intermolecular e-(γ-glutamyl)-lysine bonds to stabilize the fibrin polymer. The formation of factor XIII-catalyzed fibrin polymers during clotting of plasma and purified fibrinogen in vitro was followed by a sodium dodecyl sulfate agarose gel technique, and an increase in both amount and size of γ-chain cross-linked polymers was demonstrated before visible clot formation. Plasma from patients presenting with acute myocardial infarction showed increases in the plasma concentration of fibrin polymer and in the proportion of total fibrinogen present as polymer, as determined by a quantitative adaptation of the electrophoretic technique. The plasma concentration in patients with subendocardial or transmural myocardial infarction showed significant (p < .005) increases to 4.0 ± 1.0% and 3.6 ± .8%, respectively, as compared with the concentration in normal plasma (0.8 ± 0.1%). There was no difference in plasma concentration in samples from patients with transmural compared with those with subendocardial myocardial infarction. This study provides the first demonstration of factor XIIIa cross-linked fibrin polymers in thrombotic disease and indicates the presence of increased activity of both thrombin and factor XIIIa in patients with acute myocardial infarction.


THROMBOSIS is a pivotal event in the pathogenesis of myocardial infarction, with a 70% to 90% incidence of acute thrombotic occlusion of the artery supplying the infarcted zone in patients with transmural infarctions. In addition, the development of thrombosis overlying the infarcted area may lead to systemic embolization, and deep venous thrombosis develops in 25% of patients as detected by 125I fibrinogen scanning. The fibrin deposition that occurs with these thrombotic events is associated with changes in plasma fibrinogen that may be interpreted either as indicative of a preexisting "hypercoagulable state" or as secondary to the local thrombosis. For example, an increased action of thrombin on fibrinogen in acute myocardial infarction is reflected in an elevation in plasma fibrinopeptide A, as evidenced by increased circulating soluble fibrin identified by fibrinogen affinity chromatography, and an increase in plasma high molecular weight fibrin(ogen) complexes, as demonstrated by agarose gel filtration. In addition, elevated plasma fibrinogen has been identified as a risk factor for ischemic heart disease and for myocardial infarction, and has been associated with an increased risk of death from cardiovascular disease. Thus, insights into the structure and concentration of circulating fibrin polymers could contribute to an understanding of the pathophysiology of thrombosis in patients suffering acute coronary events.

In this report we have used a newly developed electrophoretic technique to follow changes occurring in fibrinogen during clotting in vitro and have also characterized plasma fibrinogen in patients presenting with acute myocardial infarction. After the addition of low concentrations of thrombin to purified fibrinogen or plasma in vitro, a progressive increase in cross-linked fibrin polymers was seen before visible clot formation. In patients presenting with transmural or subendocardial myocardial infarction we documented and quanti-
tated a marked increase in plasma cross-linked fibrin polymers, reflecting either a generalized hypercoagu-
able state or a systemic effect of local thrombosis.

Methods

Blood samples. After informed consent was obtained, blood samples were collected from normal volunteers who denied any history of thrombotic disease or coronary artery disease and from patients with acute myocardial infarction. Acute transmural myocardial infarction was diagnosed by the combination of persistent chest pain unrelieved by nitroglycerin, an elevated MB fraction of creatine kinase (CK), and electrocardiographic changes of persistent ST segment elevation, reciprocal ST seg-
ment depression, and subsequent development of significant Q waves. Acute subendocardial myocardial infarction was diag-

nosed by the same criteria of chest pain and enzyme elevation in patients with ST segment depression, but without development of Q waves. Infarction was localized as anterior if the electrocardiographic pattern of infarction was most prominent in the anterior leads (I, aVL, and V₆₋₉) as diaphragmatic if in leads II, III, and aVF. Frequent preexisting medical conditions at the time of admission included hypertension in 16 patients, angina in 10, diabetes mellitus in nine, and congestive heart failure in five. Other illnesses present in three or fewer patients included emphysema, Parkinson’s disease, osteoporosis, epilepsy, hypothyroidism, dementia, pernicious anemia, schizophrenia, iron-deficiency anemia, atrial fibrillation, gout, claudication, sideroblastic anemia, bladder carcinoma, and chronic renal fail-
ure. At the time of presentation, the patients were taking a wide variety of medications, including diuretics in 10, hypoglycemic drugs in seven, antihypertensive drugs in seven, nitroglycerin in seven, β-blockers in six, nonsteroidal anti-inflammatory agents in four, digoxin in four, calcium-channel blockers in four, and allopurinol in three. Blood samples were obtained within 27 hr of onset of chest pain by venipuncture of an antecubital vein or from an indwelling arterial catheter. After the first 10 ml was discarded, blood was drawn into EDTA (0.3% final concentration) and epsilon aminocaproic acid (EACA) (0.2M final concentra-
tion). Within 30 min of collection the blood was centri-
lar to that in normal blood and only a faint trace of trimer was noted. In addition, a more rapidly migrating band with a mobility consistent with that of fragment Y or DD was seen in samples 2, 3, 4, and 6, which was indicative of some fibrin or fibrinogen degradation.

To determine their polypeptide chain composition, fibrin polymers were prepared in vitro by the addition of a low concentration of thrombin (0.02 U/ml) to purified fibrinogen and this solution was characterized by two-dimensional electrophoresis (figure 2). Electrophoresis in the first dimension demonstrated the monomer and at least five polymeric forms. The second dimension analysis after disulfide bond reduction showed that the monomer separated into three bands. The Bβ and γ chains corresponded to those in fibrinogen, while the third band migrated further than the Aα chain of fibrinogen but not as far as the fibrin α chain, suggesting partial cleavage of fibrinopeptide A. The composition of the polymers was different; each showed both γ monomer and γ dimer bands with relatively more dimer and less monomer in each successively larger polymer. Residual uncross-linked α chains in all fibrin species migrated slightly further than the Aα chain of fibrinogen, reflecting cleavage of the A peptide in each polymer. However, fibrinopeptide B had not been cleaved since a band with mobility identical to that of the Bβ chain of fibrinogen was present in all polymers. These data indicate that the fibrin polymers lacked fibrinopeptide A and were covalently bound by γ chain cross-linking, with proportionately less γ monomer in each larger polymer.

To follow the progress of polymer formation during activation of coagulation, freshly drawn normal plasma was clotted in vitro by the addition of calcium and thrombin (0.025 U/ml) (figure 3). Before addition of thrombin, the normal plasma showed a faint dimer band that increased in intensity slowly after addition of thrombin and was clearly more prominent at 9 min, a time that was 0.75 of the clotting time. At 11 min (0.92 of the clotting time and 1 min before any visible fibrin

![FIGURE 2. Two-dimensional electrophoretic analysis of fibrin polymers. Fibrinogen (2 mg/ml) was incubated with thrombin (0.02 U/ml final concentration) and calcium chloride (0.05M final concentration) at 25°C for 10 min, at which time no visible gelation had occurred. An aliquot of the mixture was diluted with an equal volume of SDS-containing diluent and electrophoresed on an SDS 2% agarose gel. The pattern following Coomassie Blue staining is shown in the first dimension at the top and demonstrates a band corresponding to fibrinogen and up to five polymers. A parallel strip was cut from the gel, incubated in a reducing solution, and electrophoresed into the 7% acrylamide gel at right angles toward the anode (bottom). Samples of reduced fibrinogen (right) and crosslinked fibrin (left) are electrophoresed in the second dimension for comparison. Bands corresponding to Aα, α, Bβ, β and γ chains, γγ dimers, and the fibronectin subunit are indicated.](http://circ.ahajournals.org/content/full/86/6/1172/F2.large.jpg)
formation) the dimer was even more prominent, with at least 10 polymeric forms that could be distinctly identified; larger polymers that failed to enter the gel were also present. This pattern persisted at 13 min, a time at which small fibers could be seen, but the last sample obtained before solid clot formation (16 min) showed disappearance of the largest polymers. Without added calcium chloride and in the presence of EDTA (.3% final concentration) to inhibit the activity of factor XIIIa, there was no increase in the dimer band.

The electrophoretic technique was analyzed quantitatively by densitometry, which revealed a log-log relationship between band height and concentration that applied to either the monomer or dimer bands alone or to the summed total height of all bands (figure 4). On each gel a series of fibrinogen samples in an appropriate range of concentrations was electrophoresed, and a standard curve was constructed relating fibrinogen concentration to the total height of all bands in the sample. The amount of protein in bands in an unknown was determined by comparison with the standard curve.

Electrophoretic analysis of plasma was performed in 14 normal control subjects, 19 patients with acute transmural myocardial infarction, and nine patients with acute subendocardial myocardial infarction (table 1). The control group consisted of individuals with no known cardiac or vascular disease who had the same sex distribution but a somewhat younger age than those in the myocardial infarction group. Patients with transmural myocardial infarction had higher peak CK concentrations than those with subendocardial myocardial infarction, reflecting a larger infarct in the former group. At the time of sampling, 23 of 28 myocardial infarction patients were receiving heparin as prophylaxis for deep-vein thrombosis and three were in clinical shock.

In comparison with controls, patients with acute myocardial infarction had significantly increased fibrin polymers, as expressed by three variables (table 2). First, the ratio of the heights of dimer and monomer bands was significantly greater in both the transmural myocardial infarction group (p < .005) and the subendocardial myocardial infarction group (p < .02). Second, the absolute plasma concentration of dimer (determined from the standard curve; figure 4) was higher in both myocardial infarction groups and increased more than eightfold compared with control. Third, the proportion of dimer expressed as the percentage of total fibrinogen (determined from the standard curve) was also significantly increased in patients with transmural and subendocardial myocardial infarction (3.6% and 4.0%, respectively) compared with that in control subjects (0.8%). The proportional increase in dimer as a percentage of total fibrinogen was less than the abso-

FIGURE 3. Formation of cross-linked fibrin polymers in plasma after addition of thrombin. Thrombin (0.025 U/ml final concentration) and calcium chloride (0.05 M final concentration) were added to normal citrated plasma, and aliquots were withdrawn at the indicated times and prepared for electrophoresis followed by autoradiography. Visible fibrin strands formed at 12 min and a solid clot was present at 16 min. The "time/clotting time" value reflects the incubation time of each sample relative to the time of solid clot formation.
lute increase in dimer concentration (fourfold vs eightfold) since the plasma fibrinogen concentration was elevated in patients with myocardial infarction. There was no difference between transmural and subendocardial groups with respect to any of these variables. The individual data points (figure 5) show that for normal subjects the highest percent dimer was 1.6%. There was greater variability among patients, but only four of 28 values overlapped the normal range.

The fibrin dimer concentration was not influenced by several significant clinical variables (table 3). The mean time from onset of chest pain to sample collection was 14.9 ± 7.8 (SE) hr, with three samples obtained at less than 5 hr, eight between 5 and 10 hr, four between 10 and 15 hr, three between 15 and 20 hr, 8 between 20 and 25 hr, and two between 25 and 27 hr. There was no significant difference between the plasma fibrin dimer concentration in the 50% of samples collected before 15 hr and that in those collected after 15 hr. Linear regression analysis showed a relationship between time after onset of chest pain to collection and dimer concentration of $y = -1.1x + 144$, with a correlation coefficient of only −.07, indicating a non-significant correlation. The method of blood sampling did not affect dimer concentration, being 128 ± 32 μg/ml in the 68% of samples obtained by peripheral venipuncture and 114 ± 27 μg/ml in samples from catheters. Similarly, there was no relationship between dimer concentration and administration of heparin or

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Characteristics of control subjects and patients</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control subjects</td>
</tr>
<tr>
<td>n</td>
<td>14</td>
</tr>
<tr>
<td>Mean age (yr)</td>
<td>59.2 ± 4.0</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>8/6</td>
</tr>
<tr>
<td>Mean CK (U)</td>
<td>—</td>
</tr>
<tr>
<td>Heparin (n)$^a$</td>
<td>—</td>
</tr>
<tr>
<td>Shock (n)$^b$</td>
<td>—</td>
</tr>
</tbody>
</table>

Mean values are ± SE.

MI = myocardial infarction

$^a$Twenty-one patients received 5000 U subcutaneously every 12 hr; two patients received intravenous heparin.

$^b$Blood pressure equal or less than 90 mm Hg systolic with tissue hypoperfusion.
the occurrence of shock. At the time of presentation, none of the patients were receiving anticoagulant drugs and none had other thrombotic disease.

Discussion

Analysis of the polypeptide chain composition of soluble fibrin formed during clotting of purified fibrinogen (figure 2) showed the polymers to be cross-linked through their \( \gamma \) chains, with approximately equal amounts of \( \gamma \) monomer and \( \gamma \gamma \) chain in the fibrin dimer and proportionately less of the \( \gamma \) monomer in successively larger fibrin polymers. This pattern is compatible with formation of progressively longer linear polymers, with all \( \gamma \) chains cross-linked except for one in each of the terminal fibrin residues. There was evidence of cleavage of fibrinopeptide A in all polymers but no detectable cleavage of fibrinopeptide B, suggesting that the principal component of the polymers was fibrin 1,16,17 These results are consistent with findings of Alkjaersig and Fletcher18 indicating that soluble fibrin oligomers consist of monomers lacking both A peptides and two "fibrinogen" molecules, each lacking a single fibrinopeptide A. Our findings are also in agreement with previous reports identifying cross-linked polymers during clotting of purified fibrinogen in vitro. Sasaki et al.19 identified soluble factor XIII\(-\)stabilized dimers in mixtures of fibrinogen with low

**DIAGNOSTIC METHODS—THROMBOLYSIS**

## TABLE 2

Fibrin dimer content of plasma from control subjects and patients with acute myocardial infarction

<table>
<thead>
<tr>
<th></th>
<th>(1) Control subjects</th>
<th>(2) Patients with transmural MI</th>
<th>(3) Patients with subendocardial MI</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio of dimer/monomer height</td>
<td>0.19±0.01</td>
<td>0.37±0.03</td>
<td>0.29±0.03</td>
<td>p&lt;.005</td>
</tr>
<tr>
<td>Absolute dimer concentration (µg/ml)</td>
<td>15±3</td>
<td>123±30</td>
<td>126±39</td>
<td>p&lt;.005</td>
</tr>
<tr>
<td>Percentage of total fibrinogen present as dimer</td>
<td>0.8±0.1</td>
<td>3.6±0.8</td>
<td>4.0±1.0</td>
<td>p&lt;.005</td>
</tr>
</tbody>
</table>

All values are mean ± SE.

**FIGURE 5.** Percentage of total plasma fibrinogen represented by fibrin dimer. Data points are for 14 normal subjects, 19 patients with transmural myocardial infarction, and nine patients with subendocardial myocardial infarction. The bars indicate the mean and SE for each group (see table 2).

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## TABLE 3

Concentration of fibrin dimer in relation to significant clinical variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>Dimer (n)</th>
<th>Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time for onset of pain</td>
<td>14</td>
<td>126±27</td>
</tr>
<tr>
<td>&gt;15 hr</td>
<td>14</td>
<td>125±45</td>
</tr>
<tr>
<td>&lt;15 hr</td>
<td>14</td>
<td>126±27</td>
</tr>
<tr>
<td>Blood collection</td>
<td>19</td>
<td>128±32</td>
</tr>
<tr>
<td>Peripheral vein</td>
<td>14</td>
<td>128±32</td>
</tr>
<tr>
<td>Catheter</td>
<td>5</td>
<td>114±27</td>
</tr>
<tr>
<td>Heparin</td>
<td>23</td>
<td>122±27</td>
</tr>
<tr>
<td>Yes</td>
<td>5</td>
<td>132±41</td>
</tr>
<tr>
<td>No</td>
<td>25</td>
<td>124±26</td>
</tr>
</tbody>
</table>

\*All values are mean ± SE; no significant difference noted (p = NS) in any of these comparisons.
concentrations of fibrin, and Kanaide and Shainoff demonstrated that factor XIII could cross-link the γ chains of fibrinogen but that the cross-linking of fibrin occurred much more rapidly. Several other studies have confirmed these observations, demonstrating cross-linked oligomers before clotting of solutions of purified fibrinogen, and recently, Selmayr and Muller-Berghaus, using agarose acrylamide electrophoresis, demonstrated polymers of up to 10 units during clotting of purified fibrinogen.

Prior evidence for the presence of cross-linked polymers in plasma, as opposed to systems in vitro using purified fibrinogen, has been indirect. Ty and Jakobsen added radiolabeled fibrinogen to plasma and showed, by polyacrylamide gel electrophoresis in the absence of SDS, its conversion to slower moving bands with mobilities consistent with polymers. However, only a small proportion of these remained after electrophoresis in urea, leading to their conclusion that mainly noncovalent attractions had been present. The identification of crosslinked γ dimers in extracts of patient plasma prepared with ethanol or β-alanine, or in clots prepared in the presence of EDTA to inhibit factor XIII, action in vitro, is consistent with the presence of circulating cross-linked polymers. However, the electrophoretic systems used in these studies did not distinguish polymeric forms from high molecular weight plasmic derivatives of cross-linked fibrin, which also contain intact γ dimer chains. While we have not directly demonstrated γγ dimers in plasma, the polymers formed in vitro (figure 3) and in patient plasma (figure 1) have the same size, electrophoretic mobility, and resistance to depolymerization by SDS as those identified during clotting of purified fibrinogen (figure 2), suggesting a similar structure and derivation.

Most methods that have demonstrated alterations in plasma fibrinogen in patients with acute myocardial infarction have identified derivatives resulting from thrombin, but not factor XIII action. For example, the plasma concentration of fibrinopeptide A has been shown to be elevated in patients with acute myocardial infarction, with the highest concentration in the first 10 hr, corresponding to the time of development of thrombosis. Reinicke et al. have used fibrinogen affinity chromatography to isolate soluble fibrin from plasma in which thrombin cleavage has uncovered a binding site involved in polymerization. In patients with acute myocardial infarction they found a plasma fibrin concentration of up to 60 μg/ml, an amount increased 10-fold compared with that in normal subjects, but they did not establish whether cross-linking had occurred. Also indicative of thrombin action in acute myocardial infarction is the marked increase in high molecular weight, noncovalently bound complexes of soluble fibrin with fibrinogen and degradation products of fibrinogen and fibrin that has been demonstrated by agarose gel filtration of plasma. By contrast, our findings are most consistent with the combined action of both thrombin and factor XIII on fibrinogen in vitro. Thrombin is required to generate low concentrations of circulating fibrin monomer and also to activate factor XIII, which then cross-links γ chains of adjacent monomers after their noncovalent attraction. The demonstration that factor XIII binds to fibrin polymers in vitro and that this binding lowers the thrombin concentration required for activation of factor XIII supports the hypothesis that low thrombin concentrations could activate factor XIII in vivo, resulting in the formation of cross-linked fibrin polymers. Tissue transglutaminases are also capable of catalyzing the cross-linking reaction, but are unlikely to be involved in cross-linking of plasma proteins.

In plasma from patients with acute myocardial infarction we have documented, using a quantitative adaptation of the electrophoretic technique, a marked increase in the amount of fibrin polymer (table 2). The increase was similar in patients with transmural and those with subendocardial infarction and could be expressed as either an increase in the proportion of total fibrinogen antigen present as dimer or as an increase in absolute dimer concentration. There was considerable variability in the amount of polymer found among individual patients, as shown in the gel patterns (figure 1), large standard error of means (table 2), or individual values (figure 5). This may reflect varying degrees of activation of coagulation during coronary artery thrombosis or be caused by sampling at different times during the temporal evolution of the thrombotic process. It is unlikely that the increased plasma fibrin polymer content was caused by an artifact in vitro produced during sample processing or storage since the blood was collected into EDTA, which inhibits factor XIII and was then immediately centrifuged and diluted with a diluent containing SDS. Samples processed in this way have shown little increase in polymer content, even after prolonged storage.

The increase in circulating fibrin polymers may reflect a systemic hypercoagulable state or may result from activation of coagulation at a local site of thrombosis or tissue injury. If caused by a systemic activation of coagulation, then quantitation of circulating fibrin polymers could be used as an indicator of “hypercoagulability.” If the increase reflects changes in
the blood secondary to a local thrombotic process, monitoring of the plasma fibrin polymer could help to determine the extent and temporal course of the thrombotic process. The presence of circulating fibrin polymers also has implications for fibrinolytic therapy with newer "fibrin specific" agents such as tissue plasminogen activator that have the property of high-affinity binding to fibrin. First, in addition to binding to fibrin in thrombi, these activators may also bind to circulating cross-linked fibrin polymers, as we have demonstrated, thereby limiting their delivery to sites of thrombosis. Second, activation of plasminogen activator in association with circulating polymers could contribute to the systemic fibrinogenolysis that accompanies their use. Finally, we have previously reported a correlation between the appearance of serum cross-linked fibrin-specific derivatives and the degradation of plasma fibrin polymers. Therefore, the high concentrations of fibrin-specific derivatives identified during fibrinolytic therapy may derive more from degradation of circulating fibrin polymers than from pathologic thrombi, thereby limiting the potential for monitoring thrombolysis by following their concentration in blood.

References

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C W Francis, D G Connaghan, W L Scott and V J Marder

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