

PATHOLOGY FEATURE
WILLIAM R. HART, MD, EDITOR

The fibrinolytic system

Recent advances

FRED V. LUCAS, MD AND MICHAEL L. MILLER, DO

■ The fibrinolytic system is the subject of renewed and intense interest. As understanding of its complexity at the biochemical level has increased, appreciation of the role of this system in the pathophysiology of cardiovascular disease has grown. In addition, techniques in molecular biology have been applied to various proteins in the system to produce larger quantities of traditional and genetically engineered plasminogen activators. Many of these advances at a biochemical and molecular level will be increasingly translated into clinical medical practice and practical clinical laboratory tests. This review highlights some of these advances and the laboratory tests that may be employed.

□ INDEX TERM: FIBRINOLYSIS □ CLEVE CLIN J MED 1988; 55:531-541

HEMOSTASIS is a dynamic process generally appreciated in terms of either the coagulation system or platelet function, since much of our knowledge—until recently—has been restricted to the mechanism of thrombus formation. However, with increased understanding of the fibrinolytic system, cognizance of the role this system plays in normal hemostasis and disease states has become increasingly important. Its significance is emphasized by the attention currently given to thrombolytic therapy¹⁻³ and the fact that abnormalities of either coagulation or fibrinolysis may mimic each other clinically.

Major developments in the field of fibrinolysis have influenced the therapy of thrombotic disease, and they will be increasingly translated into practical clinical

laboratory testing. This review is intended to provide an update on these advances and some of the newer laboratory tests used to evaluate fibrinolysis.

In general biochemical terms, the fibrinolytic system is similar to the coagulation system but is functionally antithetic in that it digests fibrin and removes fibrin clot once hemostasis is achieved.⁴⁻⁷ Other functions attributed to it include tissue degradation as a basic response in a wide variety of neoplastic and non-neoplastic conditions.⁸

Central to understanding the role of the fibrinolytic system in thrombosis is the concept that physiologic clot lysis occurs due to incorporation of fibrinolytic system components into the clot during its formation. These components include both activators and inhibitors of the system, which ideally produce a balanced, controlled lytic response. Under normal circumstances, fibrinolysis proceeds slowly relative to coagulation, thus allowing an effective hemostatic response to injury. Clinical events manifested by excessive bleeding or excessive clot formation may be due to inappropriately brisk or delayed fibrinolytic responses.

At the core of the fibrinolytic system is plasminogen,

From the Department of Pathology and Laboratory Medicine, The Cleveland Clinic Florida, Fort Lauderdale, FL (F.V.L.) and the Department of Laboratory Hematology, The Cleveland Clinic Foundation, Cleveland, OH (M.L.M.). Submitted Jan 1988; accepted April 1988.

Address reprint requests to F.V.L., Department of Pathology and Laboratory Medicine, The Cleveland Clinic Florida, 3000 West Cypress Creek Road, Fort Lauderdale, Florida 33309

TABLE 1
HUMAN PLASMA SERINE PROTEASE INHIBITORS

Plasminogen activator inhibitors
α_2 -plasmin inhibitor
α_2 -macroglobulin
Antithrombin III
Heparin co-factor II
C_1 -esterase inhibitor
Inter-alpha-inhibitor
Anti-chymotrypsin
α_1 -antitrypsin
Protein C inhibitor

a zymogen that is converted via activators to the active enzyme plasmin. Hemostatic substrates of plasmin include fibrin, fibrinogen, the thrombin-activated forms of factors V and VIII:C, and platelet membrane glycoprotein Ib. Activation of plasminogen may be initiated by a variety of activators such as tissue plasminogen activator (t-PA), urokinase, or the contact system of coagulation.

The fibrinolytic response may be modulated in several ways. First, it may be inhibited by specific protein inhibitors such as plasminogen activator inhibitor (PAI), which inhibits tissue plasminogen activator, or α_2 -plasmin inhibitor (α_2 -PI), which inhibits plasmin. Second, it may be enhanced by cofactors such as fibrin, which dramatically promotes t-PA-induced activation of plasminogen. Third, it may be altered by changes in the production and release of plasminogen activator.

THE FIBRINOLYTIC SYSTEM

Plasminogen, plasmin, and plasmin inhibitors

Plasminogen is synthesized in the liver as a single-chain glycoprotein having a molecular weight of approximately 92 kd.^{6,7,9} Its plasma concentration is approximately 20 mg/dL, or 2 μ M, and it may be recovered in the euglobulin fraction of plasma together with plasminogen activators. The plasminogen molecule contains 790 amino acid residues, 24 disulfide bridges, and five triple-loop structures called "kringles," so named after Scandinavian pastry having this form. These kringles contain lysine binding sites that mediate binding of plasminogen to substrates such as fibrin. When bound to fibrin, plasminogen may be more easily converted to plasmin and the effect of the plasmin is relatively localized to adjacent fibrin.

Native plasminogen has an NH_2 -terminal glutamic acid and is thus termed Glu-plasminogen. Plasmin may convert Glu-plasminogen by limited proteolysis to modified forms having an amino-terminal lysine, valine, or

methionine. These forms are respectively termed Lys-plasminogen, Val-plasminogen, and Met-plasminogen. Either Glu-plasminogen or Lys-plasminogen may be converted to plasmin. This conversion is mediated by plasminogen activators, which will be discussed in the next section. The activation of plasminogen is achieved by cleavage of plasminogen at the arginine₅₆₀-valine bond to yield a two-chain, disulfide-linked molecule. Lys-plasminogen has a higher affinity for fibrin than does Glu-plasminogen and thus it is more rapidly converted to plasmin by certain activators such as tissue plasminogen activator. Therefore, the autocatalytic conversion of Glu-plasminogen into Lys-plasminogen by plasmin is an example of a positive feedback loop.

The two-chain, disulfide-linked, plasmin molecule is composed of a heavy chain and a light chain. The heavy chain possesses the five kringles with associated lysine binding sites that are responsible for binding plasminogen and plasmin to fibrin, tetranectin, epsilon-amino caproic acid (EACA), α_2 -plasmin inhibitor (α_2 -PI), and histidine-rich glycoprotein (HRG). The light chain contains the active serine site responsible for proteolytic activity.

As a serine protease, plasmin is subject to inhibition by a variety of serine protease inhibitors.^{6,7,10} Human plasma contains at least nine different serine protease inhibitors (Table 1). In purified systems, plasmin is inhibited by α_2 -PI, α_2 -macroglobulin, α_1 -antitrypsin, antithrombin III, and C_1 -esterase inhibitor. However, plasmin generated in plasma is primarily and rapidly bound to α_2 -PI as 1:1 complex; a smaller proportion is bound more slowly to α_2 -macroglobulin. The binding of plasmin to α_2 -PI is essentially irreversible. The α_2 -PI is incorporated into fibrin clots where it is crosslinked to the clot by factor XIIIa. It is believed that this incorporation contributes to clot stabilization by preventing uncontrolled lysis by plasmin. The concentration of α_2 -PI in plasma is approximately 1 μ M. Since the plasma plasminogen level is twice that, or 2 μ M, it follows that plasmin may be generated after α_2 -PI has been depleted. Plasmin formed when α_2 -PI is depleted may have significant effects on systemic fibrinogen levels, for example during thrombolytic therapy.

The availability of plasminogen for activation may be affected by certain plasma proteins and antifibrinolytic agents.⁶ Approximately 40% of plasminogen is bound to plasma HRG while smaller amounts are bound by tetranectin and thrombospondin. Increased or decreased binding of plasminogen by these proteins may, respectively, either dampen or enhance the fibrinolytic response. EACA and tranexamic acid are therapeutic an-

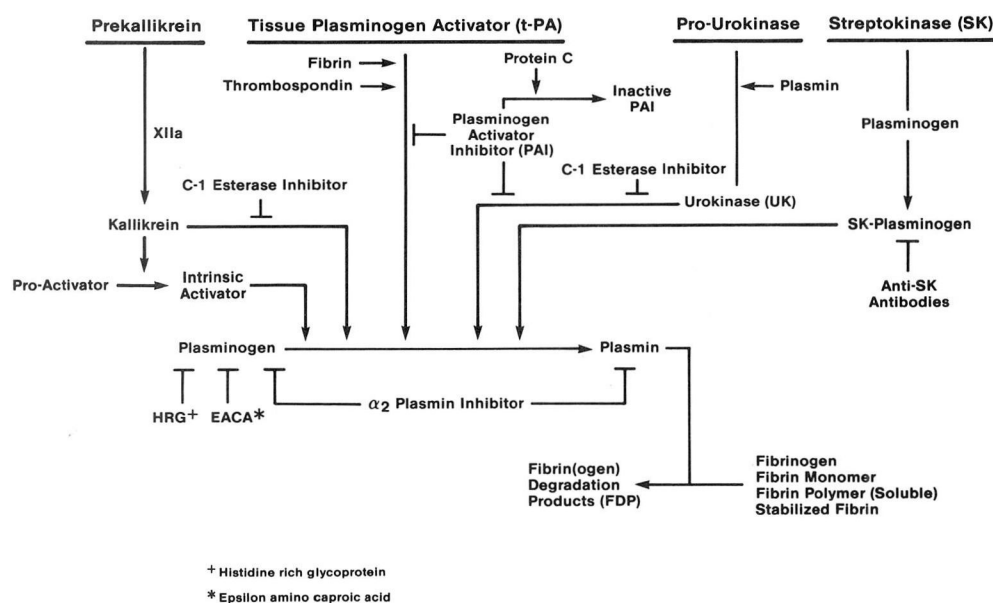


FIGURE 1. The biochemical pathways in the fibrinolytic system. A variety of activators may convert plasminogen to plasmin, which then degrades fibrin. HRG = histidine-rich glycoprotein; EACA = epsilon aminocaproic acid.

tifibrinolytic agents that bind to the lysine binding sites of plasminogen kringle, thus preventing interaction with fibrin and subsequent activation to plasmin.

Plasminogen activators and inhibitors of activation

Physiologic activation of plasminogen may be accomplished by one of three distinct pathways shown in Figure 1. These are the contact system (Hageman factor and kallikrein) dependent pathway, the tissue plasminogen activator (t-PA) pathway, and the urokinase dependent pathway. Pharmacologic activation may be achieved using either streptokinase (SK) or recombinant versions of physiologic activators.

Contact system-dependent pathway. This pathway depends on the contact system of coagulation and is inhibited by C₁-esterase inhibitor.^{4-7,11} Hageman factor, or factor XII, is converted to an activated form (XIIa) when exposed to negatively charged surfaces in association with high-molecular-weight kininogen (Fitzgerald, Fleaujac, Williams factor). XIIa is a serine protease that converts prekallikrein (Fletcher factor) to the enzyme kallikrein. Although kallikrein can, under certain conditions, activate plasminogen directly, recent evidence suggests that yet another zymogen to enzyme conversion may first be necessary. Although the importance of this activation pathway has been disputed, the clinical observation is that patients with Hageman factor deficiency do not bleed but may be at risk for thrombosis.

T-PA pathway. This pathway has received the most attention recently.⁴⁻⁷ It consists of t-PA and a rapid-acting inhibitor of t-PA, termed plasminogen activator inhibitor (PAI). T-PA has been identified in several tissues and organs, including endothelium, from which it is secreted into the circulation. The release of t-PA from endothelium can be induced by a number of substances, which include thrombin, epinephrine, bradykinin, histamine, acetylcholine, platelet activating factor, and an analogue of vasopressin, DDAVP. Exercise and venous occlusion will also induce its release. It has been

suggested that t-PA release may be under neurohumoral control via a t-PA releasing hormone from the pituitary.

T-PA has been extensively characterized and the genetic material that codes for its production, has been cloned.¹²⁻¹⁵ It is a serine protease synthesized as a single-chain glycoprotein with a molecular weight of approximately 60kd. Plasmin will convert it to a two-chain, disulfide-linked form. Both the single- and double-chain forms have enzymatic activity that is greatly enhanced in the presence of fibrin. This is because t-PA contains a finger domain and lysine binding sites in kringle structures that enable it to bind avidly to fibrin. The assembly of both t-PA and plasminogen onto a fibrin surface lowers the K_m of plasminogen activation from 65 μM to 0.2 μM, thus making the reaction far more efficient. This feature contributes to the relative clot specificity of thrombolysis by t-PA.

The half-life of t-PA in the circulation is quite short (2–5 minutes) due to hepatic clearance and binding to fibrin. The plasma concentration of t-PA may be measured at different values depending on the method used. Antigen determinations yield values between 5 and 10 ng/mL, whereas activity measurements range from 0.1–0.4 IU/mL. This ratio of activity to antigen differs from highly purified standards of t-PA, which have a specific activity of 0.5–0.8 IU/ng. The reason for the low activity in plasma relative to the antigen level is that plasma contains an excess of a rapidly acting

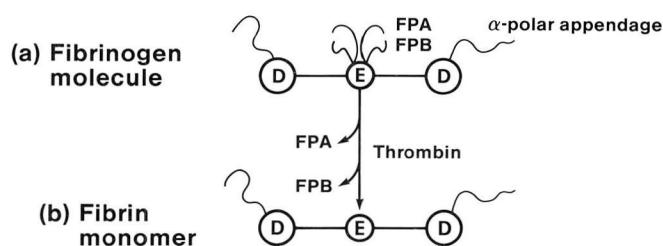


FIGURE 2. Fibrinogen is converted to fibrin monomer through the action of thrombin. Fibrinopeptides A (FPA) and B (FPB) are released.

plasminogen activator inhibitor (PAI; plasma concentration approximately 5–10 IU/mL). In all likelihood, most t-PA from blood taken under resting conditions circulates as a complex with PAI.^{16,17} Only when sufficient t-PA is released does it overcome the effects of PAI and become measurable. For this reason, it is currently believed that PAI may play a significant role in the regulation of fibrinolysis.

These are at least four immunologically distinct groups of PAI: the endothelial cell type (PAI-1), the placental type (PAI-2), protein C inhibitor, and protease nexin I.¹⁸ PAI-1 is found in endothelial cells, hepatocytes, granulosa cells, vascular smooth muscle cells, and platelet alpha-granules. PAI-1 constitutes about 60% of the total t-PA inhibitory capacity of plasma. It inhibits both t-PA and urokinase. PAI-2 is found in placenta and probably histiocytes/macrophages. It inhibits urokinase, to some extent the two-chain form of t-PA, and to a small degree the single-chain form of t-PA. Protease nexin I is a recently described broad-spectrum serine protease inhibitor that rapidly binds to thrombin when heparin is present. It also binds to single-chain t-PA and urokinase.

The exact role of the different types of PAI and the regulation of their levels remains to be clarified. PAI is incorporated into clots where it is believed to regulate the rate and extent of t-PA-induced lysis. Plasma t-PA inhibitory capacity appears to behave as an acute-phase reactant and it shows a distinct diurnal variation. The lowest values occur in mid-afternoon at a time when t-PA activity is at its peak. Stimulation of endothelial-cell PAI is produced by endotoxin and interleukin-1. Stanazolol, an anabolic steroid, lowers PAI activity in plasma when administered over a 2–3 week period. PAI may be neutralized by binding to activated protein C and this has been proposed as an explanation for the profibrinolytic properties of protein C.

Urokinase-dependent pathway. Recovered in small quantities from large volumes of urine or produced from

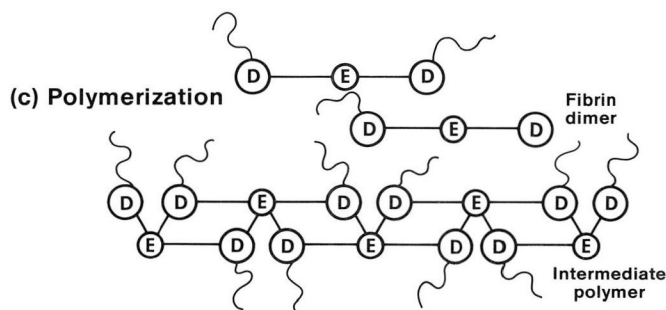


FIGURE 3. Fibrin monomer polymerizes to form higher-order forms of polymeric fibrin.

human fetal kidney cell cultures, urokinase (UK) is a disulfide-linked two-chain serine protease distinct from t-PA.^{19–22} It directly converts plasminogen to plasmin. It is initially synthesized as an inactive single-chain protein, termed pro-urokinase (pro-UK) or single-chain urokinase-like plasminogen activator (scu-PA). Pro-UK may be identified as a normal component of plasma. The conversion of pro-UK to UK is mediated by plasmin, an effect that may be stimulated by heparin.

Pro-UK appears to be relatively clot-selective in its lytic properties whereas UK will cause both clot lysis and systemic fibrinogenolysis. The mechanism for this effect of pro-UK is currently being investigated, but may be related to the fact that free plasmin is generally not present in the circulation. Both PAI and C₁-esterase inhibitor will inhibit UK; neither inhibits pro-UK.

Pharmacologic activators. The prototype activator used for therapeutic thrombolysis, SK, is a product of group C beta hemolytic streptococci.^{23,24} It activates plasminogen indirectly by binding to plasminogen. The SK-plasminogen activator complex in turn converts another molecule of plasminogen to plasmin. The SK-plasminogen complex may itself undergo plasmin-mediated conversion to SK-plasmin, which retains activator activity.

Either form of activator will exhibit activity against plasminogen regardless of the presence of fibrin. This means that plasmin will be formed both within a clot and in the circulation. The resulting changes in systemic levels of hemostatic components such as fibrinogen have been blamed for some of the bleeding complications of SK therapy, although this relationship is disputed. Patients with previous exposure to beta hemolytic streptococci will often have significant titers of antibody to SK, especially if the infection has been recent.

Because of the bleeding risk and occasional refractor-

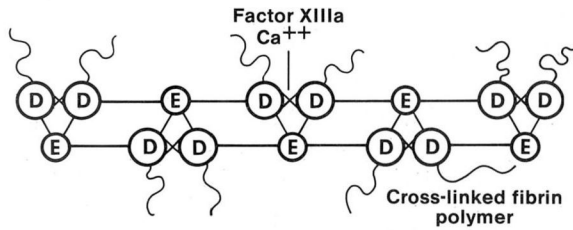
(d) Stabilization

FIGURE 4. Fibrin polymer, which is unstable, is stabilized by the action of factor XIIIa, which crosslinks adjacent molecules of fibrin.

iness to SK, therapeutic alternatives have been sought. Urokinase has been used as one alternative, but it is expensive and has been associated with similar risks of hemorrhage. Chemically modified forms of SK, acylated-SK, have been produced by attaching acyl groups to the active site of SK-plasmin(ogen) activator complexes.¹ These modified activators have fewer systemic effects on hemostasis because the acyl groups block the active site until the complex reaches the clot.

Finally, recombinant DNA technology has allowed the production of large quantities of rt-PA, r-UK, and r-pro-UK.^{14,22} All of these behave similarly to their native counterparts and therefore can be expected to exhibit similar virtues and drawbacks. The belief that a more clot-selective agent such as t-PA, pro-UK, or acylated-SK might produce less bleeding and more effective thrombolysis has driven these attempts toward new agents. Extensive clinical trials with rt-PA, r-pro-UK, and acyl-SK are underway to evaluate their safety and efficacy.

Fibrinogen and fibrin

Fibrinogen and fibrin are major substrates of plasmin that are each digested in a predictable manner, yielding specific measurable products. Fibrinogen is a symmetrical dimer (molecular weight, 340,000 d) composed of three different pairs of polypeptide chains (alpha, beta, and gamma) linked by disulfide bonds.^{7,25} The molecule is viewed as a trinodular structure by electron microscopy and is structurally divided into four regions: the carboxy-terminal (D region) domain, the central amino-terminal (E region) domain, the alpha-helical connecting coil, and the polar alpha chain appendages (Figure 2). Thrombin mediates the conversion of fibrinogen to fibrin monomer by the sequential cleavage of arginine-glycine bonds at the amino-terminal ends of the alpha and beta chains. This results in the generation of fibrinopeptides A and B (FpA and FpB). FpA is a 16-amino-acid fragment and FpB is a 14-amino-acid frag-

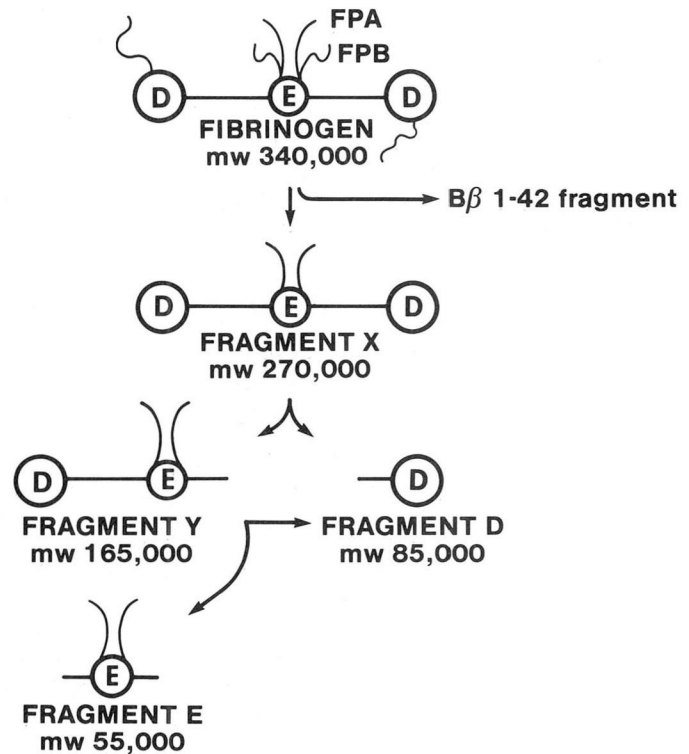


FIGURE 5. Plasmin proteolysis of fibrinogen produces a variety of smaller-molecular-weight species, termed fragment X, fragment Y, fragment E, fragment D, and the beta chain peptide B-beta 1-42.

ment. Since FpA has a short plasma half-life, approximately 1–2 minutes, elevated levels reflect very recent or ongoing thrombin generation.

Fibrin monomers produced following the removal of FpA and FpB remain in solution and will circulate in the blood as long as they constitute a small fraction of the total fibrinogen concentration. When soluble fibrin exceeds the limit it will undergo side-to-side and end-to-end polymerization to form a precipitate or clot (Figure 3). The clot is stabilized (Figure 4) by factor XIIIa, which catalyzes covalent bond formation between fibrin monomers.²⁶ Such a clot is resistant to solubilization by 5M urea or 1% monochloroacetic acid, two agents used in vitro to test for clot stabilization.

Plasmin will sequentially cleave fibrinogen (Figure 5), yielding early split products, termed fragments X and Y, and late split products, termed fragments D and E.^{7,27} Plasmin first cleaves the A-alpha chain at the carboxy-terminal end, corresponding to the polar appendages. Next the B-beta chain is cleaved at the amino-terminal end between arginine 42 and alanine 43, producing a fragment termed B-beta 1-42 and a large residual fragment termed fragment X. The B-beta 1-42 fragment is

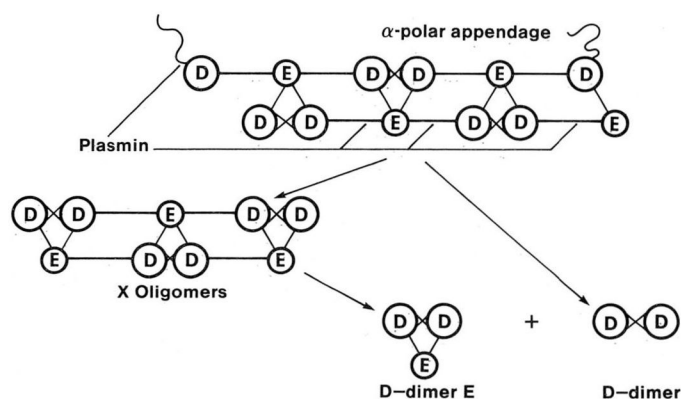


FIGURE 6. Plasmin proteolysis of stabilized fibrin yields a variety of products, some of which are distinct from those produced by degradation of fibrinogen. Two of these recently introduced as clinical laboratory tests include D-dimer and the beta chain peptide B-beta 15-42.

an indicator of the action of plasmin on fibrinogen (primary fibrinogenolysis) and it can be quantitated using an enzyme linked immunosorbent assay (ELISA) technique.²⁷ Fragment X is further degraded to fragments Y and D. The Y fragment is then split into D and E fragments (roughly corresponding to the D and E domains of native fibrinogen), which are resistant to plasmin digestion. It is the D and E epitopes that are measured in commercially available latex agglutination-fibrin split product assay bits.

The products generated from the degradation of fibrin by plasmin will vary depending on whether the fibrin clot has been stabilized by factor XIIIa. If plasmin acts on uncrosslinked fibrin, the split products will be similar to those produced during fibrinogen degradation. Plasmin digestion of a crosslinked clot (Figure 6) yields a wide variety of unique fragments, both higher and lower in molecular weight than fibrinogen. One of these is a small peptide, B-beta 15-42, which represents a product of plasmin proteolysis of the beta chain following thrombin cleavage of fibrinopeptide B. Another unique fragment, termed D-dimer, is a dimer composed of gamma-chain crosslinked D-domains of adjacent fibrin molecules.²⁷ Elevated levels of either B-beta 15-42 or D-dimer indicate action of plasmin on stabilized fibrin clot (secondary fibrinolysis).

LABORATORY EVALUATION OF THE FIBRINOLYTIC PATHWAY AND THROMBOLYTIC THERAPY

Laboratory testing in fibrin(ogen)olysis is usually directed toward one of four clinical questions: diagnosis (what is the abnormality and what is its mechanism?);

prognosis (what effect will the abnormality have?); safety (is the therapy safe?); and efficacy (will the therapy work?). Tests of the fibrinolytic system have been more problematic than those of coagulation and will probably remain so since tests are not available that offer the convenience and power of prothrombin time (PT) or activated partial thromboplastin time (APTT). However, several new ones are available to clinical laboratories that may help answer some of the clinical questions. Several of these tests require significant alterations in sample handling, but they also represent significant opportunity for interpretation.

Laboratory tests

Four types of tests may be employed (Table 2): global tests of the pathway, measurement of specific components of the pathway, markers of lytic activity, and tests to predict re-thrombosis following lytic therapy.

Global tests of the fibrinolytic pathway. These tests are all meant to indicate the overall rate or extent of fibrinolytic activity in a patient sample. They vary in complexity and sensitivity. The simplest test, whole-blood clot lysis, may be performed by drawing blood into a tube without anticoagulant, allowing it to clot, and observing the clot. Under normal circumstances the clot will remain intact for at least 24 hours, reflecting a global balance in favor of inhibition of lysis. Blood from patients with bleeding due to excessive free plasmin or plasminogen activator will exhibit clot lysis within hours. The test is charmingly simple but is probably too insensitive to be of much value except in cases of brisk fibrinolytic bleeding.

Whole-blood clot lysis may also be evaluated using a thromboelastograph, which is an instrument that imparts a mechanical force to blood placed in a stainless steel cup.²⁸ A pin placed into the blood senses both clot development and dissolution. The device can be used near the patient's bedside; lytic activity, if present, is evident within minutes to a few hours. It has recently been found useful in cases of liver transplant and surgery of large vessels.²⁹

Other uses may be made of whole blood. Clot lysis may be evaluated using clots from platelet-rich or platelet-poor plasma, though in general this approach is not much better for clinical purposes than the whole-blood technique. This is, of course, in marked contrast to the situation with the coagulation cascade wherein the PT and APTT represent significant advances over the whole-blood clotting time.

The euglobulin fraction of plasma contains fibrinogen, plasminogen activator, and plasminogen, but not

TABLE 2
LABORATORY EVALUATION OF THE FIBRINOLYTIC PATHWAY

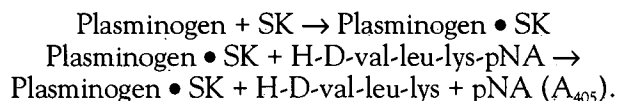
Global tests of the fibrinolytic pathway
Whole blood clot lysis
Plasma clot lysis
Euglobulin clot lysis
Fibrin plate lysis
I-125-labeled fibrin clot lysis
Thromboelastography
Measurement of specific components of the pathway
Tissue plasminogen activator
Plasminogen activator inhibitor
Plasminogen
α_2 -plasmin inhibitor
Plasmin- α_2 -plasmin inhibitor complexes
Markers of lytic activity
Fibrinogen
Fibrin(ogen) degradation products
D-dimer
B-beta 1-42, B-beta 15-42
Tests of re-thrombosis potential following lytic therapy
Fibrinopeptide A
Soluble fibrin

inhibitors of fibrinolysis. A clot formed from the euglobulin fraction will lyse readily (less than one hour) if there is excessive plasmin or plasminogen activator. Whereas a deficiency of α_2 -PI will cause rapid whole-blood clot lysis, it will not affect the euglobulin clot lysis time as readily since there is little α_2 -PI in the usual euglobulin preparation. Lytic activity may also be assessed by application of small aliquots of the euglobulin fraction to fibrin plates (plasminogen-rich to test for plasminogen activator; plasminogen-free to test for free plasmin). Zones of clearing indicate lysis and zone size correlates with the amount of lytic activity present. Since inhibitors are removed by preparation of the euglobulin fraction, lysis times or lysis zones using this fraction should be more sensitive tests for pro-fibrinolytic proteins than those using whole blood or plasma.

Measurement of specific components of the fibrinolytic pathway. The introduction of chromogenic and fluorogenic substrates has made possible the measurement of functional levels of plasminogen, α_2 -PI, t-PA, urokinase, and PAI.³⁰ Since these are enzymes and inhibitors, the assays are performed as either rate or end-point determinations. The substrates are small peptides to which chromophores or fluorophores are attached. The peptide mimics the native substrate of the enzyme being studied and color or fluorescence develops in proportion to the amount of enzyme added to the reaction mixture. Various substrates are available and instrumentation may be as humble as a simple spectrophotometer or as sophisticated as an automated centrifugal analyzer. In general, the assays are not difficult since many are avail-

able commercially in kit form.

Plasminogen is usually measured by adding excess SK to a plasma sample. This converts all the plasminogen to SK-plasminogen complexes, which have amidolytic activity against the substrate. In the simplified form shown below, the substrate is H-D-val-leu-lys-paranitroaniline (pNA) and the reaction product is monitored at 405 nm:



The assumption here, of course, is that plasminogen will function as well when physiologic activators such as t-PA are employed as it does when SK is used. The normal level for plasminogen is about 2 μM (approximately 20 mg/dL, 2.4–3.8 CTA U/mL).

The test measuring α_2 -PI uses the same substrate, but is performed by adding plasmin to the plasma sample. Available α_2 -PI in the sample will neutralize plasmin on a 1:1 stoichiometric basis. If an appropriate amount of plasmin has been added, there will be a slight excess available for amidolysis of the substrate. The result is compared with the amount of color product formed when no α_2 -PI is added to the reaction mixture. The amount by which the sample has diminished the color product compared with the blank is proportional to the amount of plasmin inhibitor present. In this case α_2 -PI is the inhibitor being measured since the time of incubation of sample and plasmin together is so short that only a rapid-acting inhibitor will be effective. The normal level for α_2 -PI is about 1 μM (approximately 80%–120% of that in pooled normal plasma).

An assay scheme similar to that for plasminogen exists for measuring t-PA and PAI and is based on recently developed t-PA-specific substrates, but most methods described have used the plasmin substrates in an indirect assay.³¹ The principle behind this approach is that plasmin substrate and plasminogen are kept in excess, solubilized fibrin is added as a cofactor, and the plasma sample is added. Color develops over the course of several hours as t-PA activates plasminogen and substrate is hydrolyzed by the formed plasmin. PAI is measured by adding exogenous t-PA to the reaction mixture and measuring neutralization of color development by PAI in the plasma sample.

There remain several difficulties with the measurement of t-PA. In baseline samples, t-PA levels are exceedingly low, approximately 0.1–0.4 IU/mL, whereas there is approximately 5–10 IU/mL of PAI. It would seem, given the excess of PAI, that t-PA should not be

TABLE 3
FIBRINOGEN – METHODS OF MEASUREMENT

Thrombin clotting
Rate – Clauss
Turbidity – Ellis and Stransky
Protein – Ratnoff and Menzies
Precipitation
Sodium sulfite
Ethanol/B-alanine
Immunologic

measurable. This problem may be partially remedied by drawing blood samples for t-PA determinations into acetate buffer at pH 3.9. The acidity neutralizes PAI since it is acid-labile, leaving t-PA free to activate plasminogen. Standardization of the fibrin used as cofactor in the t-PA assay is another difficulty. Numerous preparations from individual laboratories have been used and commercially available material is still undergoing modification.

In contrast to resting levels of t-PA, plasma levels rise appreciably when endothelial cells are stimulated by venous occlusion, exercise, or the administration of the vasopressin analogue DDAVP. Since abnormalities in this response have been described, stimulated t-PA levels are of interest, but the laboratory must be able to perform a DDAVP infusion or tourniquet arm occlusion technique in some standardized manner. The usual approach with arm occlusion has been to draw a baseline blood sample, inflate a blood pressure cuff to a point midway between systolic and diastolic pressure, leave it inflated for 15 minutes, and draw a blood sample.³² The DDAVP infusion technique has generally been to give 0.4 µg/kg intravenously for a 10-minute period and to draw pre-infusion and post-infusion samples.^{6,33} Levels of t-PA antigen after stimulation are approximately 10–20 ng/mL whereas those of t-PA activity are approximately 0.6–5.4 IU/mL. The laboratory must use caution to establish its own normal range with this technique.

Markers of lytic activity. There are direct and indirect markers of fibrinolytic activity. Plasminogen and α_2 -PI can be used indirectly to infer that lytic activity exists. During intense lytic therapy the levels of these proteins decrease predictably.^{34–38} As plasmin is generated, the level of α_2 -PI drops until it approaches 0%–10% of the pretreatment level (normally about 1 µM). At this point, plasminogen will be approximately 50%–60% of its pretreatment level (normally about 2 µM). Further administration of the lytic agent will deplete plasminogen even more. Although a similar pattern of change has been described in patients treated for acute promyelocytic leukemia (FAB M-3),³⁹ a careful search for

this pattern has not been made in more frequently encountered clinical situations such as postoperative bleeding.

Depletion of plasma fibrinogen has been more frequently used as an indirect marker of lytic activity and in fact the term "lytic state" has been applied when the level falls below 100 mg/dL. Even though serious bleeding may occur without a lytic state, measurement of fibrinogen is important because low levels have been blamed for many bleeding episodes.

There are three types of fibrinogen measurement (Table 3), based on thrombin clotting, precipitation, or immunologic procedures.⁴⁰ The method used most often in clinical laboratories is an adaptation of the Clauss method, and the materials are available in kit form. It is an example of a thrombin clotting technique that is based on the rate of fibrin formation and not on the amount of total protein in the final clot. Assessment of total protein in the clot may be achieved by chemical assay, weight measurement, or ultraviolet absorption. Precipitation techniques are based on either heat or salt (sodium sulfite, ammonium sulfate) and take advantage of the fact that fibrinogen is one of the least soluble plasma proteins. Immunologic methods include a variety of procedures—latex agglutination, immunodiffusion, immunoelectrophoresis, radioimmunoassay, and nephelometry.

The main difficulty with fibrinogen determinations during lytic therapy is that fibrin(ogen) split products (FSPs) may interfere with the measurement, and the degree of interference depends on the method used. The Clauss technique is generally claimed to be sensitive to FSPs and to give falsely low fibrinogen values.^{35,36} This may be a greater problem when a fibrometer (BBL, Baltimore, MD) is used than when the DuPont ACA (Wilmington, DE) is used.⁴¹ Precipitation techniques, on the other hand, are claimed to be less affected by FSPs and therefore may give more accurate values. The extent to which these claims are true probably varies depending on the types of FSPs present, the type of lytic agent used, and the accuracy with which FSPs are quantitated.

Until recently, tests for FSPs have been significantly limited because the antibodies used to perform the test could not distinguish between degradation products of fibrin and those of fibrinogen. Split products of fibrin would be evidence of secondary (physiologic) fibrinolysis, which accompanies disseminated intravascular coagulation, the postoperative state, or deep vein/peripheral arterial thromboembolism. Split products of fibrinogen, however, would be evidence of primary fibrinogenolysis,

a condition in which pathologic proteolytic activity exists and is directed at circulating fibrinogen.

Antibodies are now available that specifically recognize fibrin degradation products. One that has been extensively investigated and is now available to clinical laboratories recognizes the D domains of fibrin after they have been crosslinked by factor XIII and digested by plasmin.^{42,43}

The test for D-dimer can be performed either as a latex agglutination procedure or as a more sensitive ELISA technique. Since the antibody does not react with fibrinogen, plasma samples may be used for the test, obviating the need for specially preserved serum samples that are currently needed for fibrin(ogen) split product determinations. D-dimer levels are well below 1 µg/mL in normal individuals and may range up to 32 µg/mL during intense lytic therapy or in patients with secondary fibrinolysis. This is in distinct contrast to values obtained by serum fibrin(ogen) split product assays, which may range up to 1–2 mg/mL. Such readings may well be artifactually high due to residual poorly clottable fibrinogen in the serum. They may also be due to different sensitivities of serum assays produced by different manufacturers. Of the two most popular ones available, one is more sensitive to fragments D and E and less sensitive to fragment X and fibrinogen than the other.⁴³

Other antibodies to fibrin degradation products are available, but have been used less in the clinical laboratory. Plasmin degradation of fibrin yields the beta-chain peptide B-beta 15–42 whereas degradation of fibrinogen yields B-beta 1–42.^{7,27} Since these fragments are measured by ELISA, the technique is extremely sensitive (lower limit about 4.5 pmol/mL). It unfortunately requires preparation of a fibrinogen-free sample using bentonite precipitation, ethanol precipitation, filtration, or heat denaturation.

Elevated levels of both the 1–42 and 15–42 fragments during lytic therapy suggest that both fibrin clot and circulating fibrinogen are being degraded. Elevated levels of B-beta 15–42 following cessation of t-PA administration and long after t-PA has cleared from the circulation suggest sustained clot lysis due to t-PA incorporated in the clot. This effect is less apparent when SK is used. Indeed, a greater magnitude and duration of elevation of D-dimer and B-beta 15–42 have been observed with t-PA compared with SK, suggesting more intense and persistent fibrinolysis with t-PA.⁴⁴

Tests to predict re-thrombosis following lytic therapy. Following lytic therapy there is a significant risk of re-thrombosis. Tests to predict thrombosis (hypercoagulability) or the potential for re-occlusion have been

numerous and have been largely unsuccessful except in cases of familial abnormalities. However, two, for fibrinopeptide A and soluble fibrin, hold promise.^{45–49} Fibrinopeptide A may be measured by radioimmunoassay or ELISA and is normally less than 2 ng/mL in plasma. Elevated levels have been observed in a wide variety of situations such as disseminated intravascular coagulation, cardiac surgery, unstable angina, myocardial infarction, peripheral arterial or venous thromboembolism, and following lytic therapy. In fact, the levels rise during infusion of t-PA, suggesting that either thrombin in the clot is liberated into the circulation or the antibody used in the test is cross-reacting with some other species of fibrin(ogen). Rapid re-thrombosis of coronary arteries has been associated with elevated FpA levels following t-PA, despite the administration of heparin.

The attempt to measure circulating soluble fibrin has been long and difficult. The protamine sulfate test and others like it (ethanol gelation) have not been particularly successful. An electrophoretic technique recently developed at The Cleveland Clinic Foundation⁴⁹ and applied to patients with peripheral arterial occlusion suggests that soluble fibrin persists in these patients following t-PA infusion. The technique is currently being evaluated for clinical utility.

Clinical application of laboratory tests

Monitoring thrombolytic therapy. This is a controversial subject because the mechanism of the main side effect, bleeding, is not really understood. Bleeding could be due to depletion of systemic fibrinogen or it could just as plausibly be due to the inability of lytic agents to distinguish between pathologic clots (e.g. in a coronary artery) and physiologic clots (e.g. at a venipuncture site). If the latter mechanism is the more significant one, then there is little justification for measuring plasma levels of fibrinolytic system constituents. Although extensive testing is performed at the Cleveland Clinic for trials of rt-PA (*Table 4*), the main test required, from a practical standpoint, is for fibrinogen. Clinical decisions to stop lytic therapy or to administer transfusion are based only partially on the fibrinogen level and even then only when the level drops below 50 mg/dL (Clausen method-fibrometer).

If extensive testing is performed, there are significant problems with sample handling.^{35,37} Sample preservation is critical because in vitro effects of the lytic agent may occur. Protease inhibitors such as aprotinin (Trasylol) and PPACK (D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone) have been used with some success. Monoclonal antibodies to t-PA have also been

TABLE 4
LABORATORY TESTS PERFORMED AT CLEVELAND CLINIC IN
PATIENTS RECEIVING rt-PA

PT
APTT
Thrombin time
Fibrinogen – Clauss, sodium sulfite
Plasminogen
α_2 -Plasmin inhibitor
Fibrin(ogen) split products
D-dimer
B-beta 1-42
B-beta 15-42
Soluble fibrin
Fibrinopeptide A
t-PA – antigen, functional
Plasminogen activator inhibitor

PT = prothrombin time; APTT = activated partial thromboplastin time.

used successfully. It has generally been preferable to test samples before freezing them, though of course this isn't always convenient. An accurate assessment of FpA levels requires extraordinary attention to venipuncture technique, knowledge of previous intravenous lines or venipunctures, and use of protease inhibitors in the sample tubes. Spurious elevations in plasma FpA due to artifact of sample handling or the presence of indwelling catheters have been a significant problem.

Diagnostic testing. Abnormalities of the fibrinolytic system that cause bleeding (Table 5) are deficiency of α_2 -plasmin inhibitor, deficiency of plasminogen activator inhibitor, or excess plasminogen activator.^{4,6} Although not typically considered as part of the fibrinolytic system, factor XIII deficiency will cause bleeding since the clot that forms is unstable and is more susceptible to plasmin degradation.²⁶ The usual screening test for factor XIII, clot stability in monochloroacetic acid, is not especially sensitive to factor XIII deficiency, nor is it specific since it will be abnormal in α_2 -PI deficiency.

Abnormalities of the fibrinolytic system that cause thrombosis (Table 6) include plasminogen deficiency (either low antigen level or defective molecule), t-PA

TABLE 5
ABNORMALITIES OF THE FIBRINOLYTIC SYSTEM ASSOCIATED
WITH HEMORRHAGE

α_2 -plasmin inhibitor deficiency
Plasminogen activator inhibitor deficiency
Excess plasminogen activator

TABLE 6
ABNORMALITIES OF THE FIBRINOLYTIC SYSTEM ASSOCIATED
WITH THROMBOSIS

Plasminogen deficiency
Dysplasminogenemia
t-PA deficiency
Excess plasminogen activator inhibitor

deficiency, and excess PAI.^{4,6} A deficiency of t-PA following an appropriate stimulus such as DDAVP may be due to a defect in production/release, in which case the antigen level will be low, or it may be due to a functional deficiency. Functional deficiencies of t-PA may be due to excess PAI. High levels of PAI have now been described in association with deep vein thrombosis, myocardial infarction, hyperlipoproteinemia, and non-specific acute illnesses.^{4,6,39,50-52} The relationship of PAI to the mechanism of these various disorders is still being investigated.

SUMMARY

The complexity of the fibrinolytic system is now known to rival that of the coagulation cascade and platelet function at a biochemical level. Its clinical importance in cardiovascular disease is underscored by recent attempts to dissolve thrombi and ongoing efforts to define abnormalities responsible for either hemorrhage or thrombosis. Laboratory tests to study fibrinolysis have advanced significantly in the past ten years and now allow better characterization of biologic samples. Ingenuity in applying these measurements to disease states will determine how useful they are to patient care.

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