Brief Review

Sol Sherry Lecture in Thrombosis
Research on Clot Stabilization Provides Clues for Improving Thrombolytic Therapies

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This article is a summary of the Sol Sherry Lecture of the Council on Arteriosclerosis, Thrombosis, and Vascular Biology, which was presented at the 71st Scientific Sessions of the American Heart Association in November 1998.1 It highlights the work from our laboratory, designed to dissect the intricate reactions and molecular control mechanisms that operate in the final stages of the coagulation cascade. This research brought forth the idea that, by selectively blocking the maturation and accretion of thrombi, we should be able to achieve a much safer and more efficient thrombolysis at lower dosages of clot dissolving agents than currently in use.

Fibrin is the fundamental building block of the clot matrix. Network formation occurs in an orderly sequence, well separated in time into distinct phases during the course of coagulation of normal plasma. After the reaction of thrombin with fibrinogen, a protofibrillar lattice is formed, with fibrin units lined up in a half-staggered array, reminiscent of laying bricks without mortar (Figure 1, top panel). Lateral bundling into filaments and fibers with concomitant entanglements and branching generates a 3D gel, the appearance of which is a measure of “clotting time.” Then, under the influence of the activated fibrin stabilizing factor (factor XIIIa), covalent bonds are introduced into the structure that causes an irreversible, end-to-end fusion of the fibrin particles (Figure 1, middle panel). Finally, full maturation of the network is brought about by forming covalent bonds between the protofibrils and filaments (Figure 1, bottom panel). Clots displaying the features shown in the top panel of Figure 1 can be readily dissociated into monomeric fibrin in 5 mol/L urea2,3 and on removal of urea, the gel reforms. The equilibrium between the monomeric or low oligomeric forms of soluble fibrin and clotted fibrin can be described as:

\[ \text{Fibrinogen} \xrightarrow{\text{Thrombin}} \text{Fibrin} \xrightarrow{5 \text{M urea}} (\text{Fibrin})_n \text{clot} \]

In contrast to this, the factor XIIIa-stabilized networks, shown in the middle and bottom panels of Figure 1, cannot be dispersed in urea. (As a historical aside, it is interesting to observe that the question of whether fibrin could be dispersed in urea or not has been investigated and debated in the literature for more than 100 years [see reference 4], with some researchers answering in the affirmative, others in the negative. In view of what we now know,2,3 both sides may have been right on this issue).

An appropriate therapeutic aim for helping individuals with thrombotic tendencies would be to block the factor XIIIa-mediated reactions in a well controlled and highly selective manner without interfering with the primary “clotting time.” This would result in preventing a significant portion of the clot from progressing to the fully stabilized state (illustrated by the bottom panel of Figure 1).

The Urea-Soluble Clot

The dramatic conversion of fibrinogen to fibrin is caused by the proteolytic action of thrombin with the release of N-terminal fibrinopeptide moieties5–8 (Figure 2a). These “caps” have to be removed from the protein for unmasking the self-assembly potential that was built into the parent fibrinogen molecule by evolution. The demonstration that thrombin was a protease provided the first example of a limited proteolytic type of control in the now familiar mode of regulation for most of the reactions of the coagulation and immunocomplement cascades. A key feature of the reaction of thrombin with fibrinogen is the generation of N-termini of glycine in fibrin9–11 because the immediate sequences of amino acids at these newly exposed sites12 can serve as ligands for promoting the noncovalent assembly of fibrin molecules. Short peptide analogues of the natural sequences have been found to inhibit the clotting of purified fibrinogen.13

The fibrinopeptides (A and B) are located at the ends of the Aα and Bβ chains of fibrinogen in the central E domain of the large molecule (Mw~340 000), made up of a disulfide bonded duplex of 3 different chains [AαBβγ].14 Release of fibrinopeptides by thrombin sets the train of events in motion for clotting by allowing the newly unmasked N-termini to interact with complementary holes (polymerization pockets) in the distal D domains of the protein.15–17 Actually, as seen from the activities of some snake venom enzymes, the release...
of fibrinopeptides A from the Aα chains alone is sufficient to initiate clotting. In either case, the E domain of one fibrin molecule makes contact with the D domains of 2 adjacent fibrins, neatly aligning them end-to-end (Figure 2b). This mode of assembly gives rise to the half-staggered arrangement of fibrin units in the protofibrils, recognized in the electronmicroscope with characteristic ca. 230Å repeats. Although prior thrombin cleavage is necessary for unmasking the N-terminal ligands, the polymerization pockets into which these knobs fit are fully accessible also in the fibrinogen molecule. Long filamentous assemblies can be obtained by mixing fibrinogen with fibrinopeptide-denuded E fragments derived from the plasmin digest of fibrin.

The Urea-Insoluble Clot

There is a large family of Ca2+-dependent enzymes, widely distributed in nature and commonly referred to as transglutaminases, which promote the cross-linking of proteins by intermolecular N(γ-glutamyl)lysine side chain bridges. As first documented for the clotting of blood in crustaceans and also for the process of forming the copulation plug in rodents, such transamidases may have served as the evolutionary prototypes of clotting enzymes. In the clotting of lobster blood, the enzyme is released from exploding amoebocytes, performing a platelet-like function. In the clotting of the seminal vesicle secretion proteins of rodents, the transglutaminase is secreted from another lobe of the prostate and becomes activated only in the vagina of the female as it encounters its substrates after ejaculation. In both phenomena, proteins are remodelled in a single enzymatic step, resulting in their transfer directly from a soluble phase into an insoluble coagulum, extensively cross-linked through covalent bonds. In sharp contrast, with vertebrate blood, cross-linking follows clotting and the factor XIIIa-catalyzed reaction is superimposed on a preformed assembly of an aligned and organized fibrin network. Thus, in the clotting of human blood, cross-linking contributes exclusively to the maturation and rigidification of the gel but does not significantly alter the morphology of the structure.

The covalent cross-linking of fibrin units occurs through an amide exchange reaction (transamidation) between select glutamine and lysine residues of neighboring protein molecules (Figure 3a). As a result, a few strategically located N(γ-glutamyl)lysine side chain bridges are formed.

Figure 2. a) Generating the set of new N-termini of glycine in the limited proteolysis of fibrinogen by thrombin is the key for initiating the actual clotting reaction. The site of attack by thrombin in the critical step of releasing the fibrinopeptide A moiety from the Aα chain of the protein is shown. b) Release of fibrinopeptides (arrowheads) from fibrinogen triggers the process of self-assembly. The new N-terminal knobs, unmasked by thrombin in the central E domains bind to complementary holes in the D domains of the protein. Thus the E domain of 1 fibrin molecule makes non-covalent contacts with the D domains of 2 adjacent fibrins, aligning them end-to-end.

Figure 1. Stages in the formation and maturation of fibrin clots. Clotting time is defined by the rate of assembly of the protein into structures as illustrated in the top panel. Such clots, obtained in mixtures of fibrinogen or Ca2+-chelated (citrate, ethylenediaminetetraacetic acid) plasma with thrombin, can be reversibly dissociated into fibrin monomers, proving that these clots—in contrast to the covalently linked polymers in the urea-insoluble structures shown in the middle and bottom panels—are made up of fibrin molecules held together only by weak secondary bonds.
sake of simplicity, only 1 of the 2 bridges connecting the γ chains of 2 fibrins with an end-to-end orientation is shown in the figure. One should bear in mind, however, that factor XIIIa reacts with the γ, as well as the α, chains of fibrin in an ordered sequence. Crosslinks spanning more than 2 γ chains and several α chains, giving rise to a variety of γi, γj, γk structures, homologous αn and hybrid αn γi chain combinations, play important roles in clot stabilization. Furthermore, the factor XIIIa reaction is also responsible for the covalent attachment of a fraction of the α2 plasmin inhibitor (α2PI, also referred to as α2 antiplasmin or α2AP) in plasma to the α chains of some of the fibrin molecules, providing added protection against lytic agents.

Activation and Regulation of the Factor XIII System

As in the case with all other enzymes of the coagulation cascade, only the inactive precursor, ie, factor XIII, of the cross-linking enzyme circulates in plasma. The zymogen has an A2B2 subunit structure, reminiscent of the heterologous tetrameric composition of hemoglobin. The A subunits possess the catalytic potential and, quite likely, the carrier B subunits serve the function of protecting them in the circulation. Thrombin and Ca2+ are required for conversion into the active factor XIIIa enzyme, but the thrombin-catalyzed cleav-
age near the N-termini of the A subunits only weakens the heterologous association of the subunits and still leaves the zymogen in an inert tetrameric (A$_2$B$_2$) form (Figure 4). Expression of enzyme activity depends on the Ca$^{2+}$-facilitated dissociation of A$_2$B$_2$ from the carrier B$_2$ subunits and on a concerted conformational change that unmasks the hidden sulfhydryl catalytic centers for the A$_2$* (FXIIIa) cross-linking enzyme. Although the other trypsin-like enzymes of the coagulation cascade operate with serine-OH active centers, factor XIIIa functions with a cysteine-SH catalytic residue, assisted by the imidazole ring of a histidine. There are remarkable kinetic and structural similarities with the papain-calpain family of enzymes, suggesting a common evolutionary ancestor.

Correct timing for the activation and operation of the factor XIII system is of critical significance for efficient hemostasis. This is reflected in the remarkably complex and tightly controlled series of interacting mechanisms that regulate the rate of conversion of the factor XIII zymogen, as well as the rate of cross-linking of fibrin by the activated enzyme. A key feature in synchronizing the clotting and cross-linking events is that thrombin plays the dual role of converting fibrinogen to fibrin and also initiates the process of activating the factor XIII zymogen. In addition, it has become evident that the fibrin substrate itself acts to coordinate the orderly sequence of reactions during the late stages of coagulation; as a feed-forward regulator, 1) it accelerates the cleavage of factor XIII by thrombin, and 2) it enables the subunits of the thrombin-modified zymogen to dissociate at the 1.5 mmol/L concentration of Ca$^{2+}$ in plasma, and 3) the noncovalent assembly of fibrin units speeds up the end-to-end fusion of the γ chains in the D domains of the protein. These unique controls (Figure 5) must have evolved to ensure that in the physiological sequence of events, only fibrin, and not the parent fibrinogen molecule, should be the target for cross-linking by factor XIIIa.

### Determinants of the Morphological and Rheological Properties of the Clot Network

In the physiological milieu of pH, ionic strength, and Ca$^{2+}$ concentration in plasma, clot morphology is determined mainly by the concentration of fibrinogen and by the thrombin activity generated at the site of injury. In addition, some proteins adhering to fibrin, eg, IgG and albumin, may add to fiber thickness. With all other conditions being equal, scanning electronmicrographs show considerably tighter networks—characterized by greater fiber and branch point densities—near the upper end of the physiological range of fibrinogen concentration. Also, clots formed rapidly at higher concentrations of thrombin (with shorter clotting times) give rise to much tighter structures than their slowly forming counterparts generated at lower concentrations of thrombin (with longer clotting times). Tightly knit clots would, of course, increase the likelihood of a higher degree of entrapment of blood cells in the network.

Remarkably, stabilization by the factor XIII system does not seem to affect clot morphology in regard to fiber and branch point densities, but the fibers become somewhat thinner and longer, suggesting that the covalent bonds introduced by factor XIIIa strengthen the internal archite-
tures of the fibers themselves. Notwithstanding the morphological similarities, the properties of the network are greatly different because the clot can no longer be dissolved in 5 mol/L urea. Another sign of the profound change induced by the action of factor XIII is the large increase in the viscoelastic (storage) modulus, a measure of clot stiffness. To some extent, this quantity also correlates with the concentrations of fibrinogen and thrombin, but the role of factor XIII is paramount in this regard, causing an approximately 5-fold increase throughout the physiological range of fibrinogen concentration.

What Lessons Can be Learned From Studying Disorders of Fibrin Stabilization?

Hemorrhaging may occur in some individuals in spite of the finding that the clotting and primary bleeding times are within the normal range of values. The hemostatic plug in these patients seems to fail because of extreme fragility and gives rise to a delayed oozing of blood at the wound site. Diagnosis usually rests on the observation that the recalcified plasma clot of the patient, unlike a normal one, can be solubilized in 5 mol/L urea. The hereditary lack of functional factor XIII zymogen is responsible for the majority of such cases, and most of them are due to the absence of the catalytic A subunits, although cases are also known with absence of B subunits. Acquired inhibitors constitute yet another class with quite diverse etiologies in this family of disorders of fibrin stabilization. The natural inhibitors may interfere with 1 or more of the biochemical steps involved in the activation of the factor XIII zymogen (A₂B₂ → A₂⁺B₂ → A₂⁺B₂*) or with the actual production of the covalent N(γ-glutamyl)lysine crosslinks catalyzed by the A₂⁺, factor XIIIa enzyme. Most of these inhibitors are found to be autoimmune antibodies directed against some of the molecules participating in the physiological sequence of events depicted in Figure 5.

In addition to the abnormal solubility in urea, the patients' clots are much softer than normal showing very low viscoelastic moduli and a greatly enhanced susceptibility to digestion by lytic agents. These features can be illustrated with the experiments in Figure 6, carried out with factor XIII-deficient plasmas. Panel A shows that supplementation with the purified A₂B₂ zymogen restored normal (approximately 5-fold) values to clot stiffness, and the data in panel B demonstrate that supplementation with the zymogen (using only the recombinant A₂ subunits in this experiment) can also protect the clot against premature lysis (see also reference 80). These findings prove that proper functioning of the factor XIII system is required for imparting normal stiffness and adequate fibrinolytic resistance to the clot network.

Therapeutic Possibilities

Detailed understanding of the molecular aspects of clot stabilization, together with the patient studies, provide the background for rational approaches aimed at exploring means for reducing the stiffness and lytic resistance of clots and thrombi. Results of some prototype experiments, employing cross-linking inhibitors such as discussed earlier (see Figure 3b) are presented in Figure 7. In a way, the findings are the mirror images of the data in panel A of Figure 6 because, as illustrated in Figure 7 (panel A), the inhibitors can actually reduce normal clot stiffness to approximately 20% of normal, corresponding to the value found in factor XIII-deficient plasma. It is even more significant that the color photographs in panel B of Figure 7 provide decisive evidence that the specific inhibitors can also greatly enhance the susceptibilities of thrombi for digestion by lytic agents. These thrombi were formed from whole human blood in rotating plastic tubes (Chandler loop) in the absence and presence of a cross-linking inhibitor. They were indistinguishable before the addition of urokinase, but after 2 hours of exposure to the plasminogen activator, only a minuscule residue remained of the thrombus that was formed in the presence of the inhibitor, which was in

Figure 6. Supplementation of factor XIII-deficient plasma with the missing factor can normalize values for clot stiffness (panel A) and increases resistance to lysis (right hand panel). Purified A₂B₂ plasma factor XIII (0.2 to 10 × 10⁻⁹ mol/L) was used in the experiments in panel A, whereas the recombinant A₂ subunits of the zymogen were used (0 to 7 μg A₂) in panel B. The 0.2 mL mixtures for the latter comprised 0.1 mL of factor XIII-deficient citrated plasma, 0.6 U of thrombin, 12 mmol/L CaCl₂, 25 mmol/L Tris-HCl buffer pH 7.5, and 20 IU TPA at 22°C (Lorand and Velasco, unpublished data, 1999).
more possibilities for enhancing the fibrinolytic susceptibilities of thrombi.\textsuperscript{90} The Arg364Ala mutant, although no longer a plasmin inhibitor, competes effectively against the factor XIIIa-catalyzed incorporation of the wild type of $\alpha_2$PI into fibrin. This approach is a special version of the competitive blocking of the donor cross-linking sites in fibrin, as depicted by reaction 3 in Figure 3b.\textsuperscript{10}

The concept of utilizing inhibitors of clot stabilization as presented in this article provides a basic framework for developing new methodologies that, if translated to clinical practice, could facilitate thrombolysis with much lower doses of plasminogen activators than currently used. Significantly, as clotting time would not be lengthened, the extra risk of hemorrhage could be avoided.

Figure 7. Inhibitors of the factor XIIIa-catalyzed crosslinking reactions greatly reduce clot stiffness (panel A) and also the lytic resistance of thrombi (panel B). The ordinate of panel A indicates clot stiffness at 60 minutes ($G_1$,60) in the presence of crosslinking inhibitors, as percentages of the control value ($G_0$,60) without inhibitors. The color photograph in panel B shows that, after 2 hours with 300 CTA units of urokinase (UK) at 37°C, the thrombus formed in the presence of 1 mmol/L tosylcadaverine, an inhibitor of crosslinking (XL), is completely lysed (d) whereas the control specimen (b) is still quite undigested. Photographs (a) and (c) are control thrombi without urokinase.

stark contrast to the essentially undigested thrombus formed in the absence of the inhibitor.

The validity of the notion that interference with any aspect of fibrin stabilization would produce similar effects is supported by numerous other experiments. Active center directed inhibitors of factor XIIIa were shown to be highly effective in reducing the viscoelastic moduli of clots\textsuperscript{38} and also for the enhancement of clot lysis.\textsuperscript{45} A protein isolated from the giant amazon leech was described to possess similar properties, although its mode of interaction with factor XIIIa is not yet known.\textsuperscript{81–83}

Positive results were obtained with a monoclonal antibody directed against the thrombin cleavage site of factor XIII, which blocked the activation of the zymogen.\textsuperscript{84} Another interesting approach utilizes a monoclonal antibody against $\alpha_2$PI.\textsuperscript{85} As mentioned before, the factor XIIIa-catalyzed covalent attachment of $\alpha_2$PI to fibrin significantly contributes to lytic resistance.\textsuperscript{40,41} In fact, the hemorrhagic manifestations in patients with $\alpha_2$PI deficiency are being attributed to the excessive digestibility of the clot.\textsuperscript{86} The antibody to $\alpha_2$PI was shown to be effective in promoting clot lysis in vitro and thrombolysis in vivo in animal models.\textsuperscript{85,87–89} [Added in proof: Recent results from a mutant form of $\alpha_2$PI point to

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