Can Plasminogen Activators Be Improved?

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The widespread application of plasminogen activators in the treatment of myocardial infarction has resulted in a significant reduction in mortality.\(^1\) Most patients have been treated with two very different drugs: streptokinase, a bacterial protein that is the product of fermentation technology dating from the 1930s, and recombinant tissue-type plasminogen activator (rt-PA), a human protein that is one of the first widely used fruits of molecular biology. A recent study comparing streptokinase and rt-PA, presented by the Gruppo Italiano per lo Studio della Streptochinasi nell'Infarto Miocardico (GISSI) investigators at the 39th Annual Scientific Session of the American College of Cardiology (March 18–22, 1990, New Orleans, La.), showed little difference in the efficacy or major side effect profile of these two agents. (Elements of the trial's design, however, have been subjected to criticism.) Other plasminogen activators studied clinically include the human proteins urokinase and its zymogen, single-chain urokinase-type plasminogen activator (scu-PA), and a chemical derivative of the bacterial protein streptokinase, anisoylated plasminogen streptokinase activator complex (APSAC). Even though the available clinical studies do not permit a definitive choice among these five agents, it is clear that all are subject to the dual problem of limited efficacy and hemorrhagic complications. From 15% to 20% of coronary thrombi cannot be lysed, as much as 25% of successfully recanalized vessels rethrombose, 6–10% percent of patients have a major hemorrhage requiring transfusion, and approximately 0.5% of those treated suffer an intracerebral hemorrhage, generally with disastrous consequences.\(^1\)–\(^3\)

The initial promise of rt-PA was in its fibrin selectivity. The hope was that the specificity of rt-PA for this component of the thrombus would increase the efficacy of treatment and avoid hemorrhagic complications by reducing the systemic destruction of clotting factors and platelet receptors. rt-PA did prove highly selective for fibrin in experimental animals. Unfortunately, when rt-PA is administered to patients at doses sufficient to recanalize coronary arteries, it often causes the consumption of plasmin-clotting proteins. To date, there is little evidence of a reduction in hemorrhagic complications with rt-PA.

Many modifications have been made to the rt-PA molecule by recombinant DNA techniques in attempts to produce a more potent or more fibrin-selective plasminogen activator. None has met with significant success.\(^4\) A quite different approach is to target the plasminogen activator to a thrombus by using a highly selective antibody to a component of the clot. The purported advantages of this approach include a higher affinity for the ligand (antibodies often have nanomolar or subnanomolar affinities for their substrates in contrast with the 0.1-μM affinity of t-PA for fibrin), the ability to endow nonspecific plasminogen activators with fibrin affinity, and the ability to target components of the thrombus other than fibrin. Although antibody-targeted molecules have been shown to be both more potent and more selective than native plasminogen activators, in the absence of appropriate animal models only clinical testing can determine whether these advantages will translate to more efficient recanalization or fewer bleeding complications.

In this issue of *Circulation*, Collen et al\(^5\) describe a significant advance in antibody targeting. Using a conjugate of scu-PA and an antibody directed against fragment D-dimer of cross-linked human fibrin, they observed a threefold increase in potency (in comparison with native scu-PA) in a baboon femoral vein thrombosis model. The authors convincingly show that this enhancement is related to antibody targeting rather than to a difference in the pharmacokinetics of the conjugate. Although Runge et al\(^6\) previously demonstrated the efficacy of an analogous conjugate in vivo, the Collen study is important for its use of a human antibody in a primate model. The rabbit, used in the earlier study, possesses a thrombolytic system quite different from that of humans and nonhuman primates. The antigenic dissimilarities in fibrin often require that a human thrombus be studied in the circulatory system of another species.\(^5\)\(^,\)\(^7\)

To effectively target fibrin but not fibrinogen, the antibody must be directed to a fibrin-specific epitope. In their report, Collen et al\(^5\) chose to use the D-dimer as target epitope. A product of fibrin cross-linking by the transglutaminase factor X\(\text{IIa},\) the D-dimer is thought to be resistant to fibrinolysis. In earlier work on antibody-targeted plasminogen activators,
researchers at my laboratory took advantage of another fibrin-specific epitope, the amino terminus of the β-chain, which becomes available for antibody binding after thrombin cleaves fibrin to release fibrinopeptide B.8-10 Because it was readily susceptible to cleavage by plasmin, there had been concern that the β-chain epitope would be lost early from a thrombus during the course of its dissolution. More recent work, however, has shown that this epitope is lost from fibrin at a rate similar to the rate of overall fibrin fragmentation (F. Chen, G.R. Matsueda, unpublished observations). Comparable in vivo thrombolytic studies (in the rabbit) of rt-PA chemically linked to the β-chain-specific antibody6 and scu-PA chemically linked to the D-dimer-specific antibody11 showed no great difference between the two conjugates.

Other antigens of interest in the thrombus include platelet surface receptors and α2-antiplasmin. It has been postulated that platelet-rich clots are particularly resistant to thrombolysis and that they may account for a significant fraction of reperfusion failures.12 Gold et al12 showed that antiplatelet GPIIb/IIIa receptor antibody 7E3 enhanced the action of rt-PA in experimental platelet-rich thrombi. Clinical trials of 7E3 have now begun. In preliminary studies, Bode et al13 showed that a conjugate of urokinase and antibody 7E3 was a more potent thrombolytic agent than an equimolar mixture of the two proteins. Presumably, the antibody targets urokinase to the GPIIb/IIIa receptor. It remains to be determined whether the enzyme attacks the receptor itself or receptor-bound fibrinogen, which serves to link platelets to one another.

The concentration of the principal inhibitor of plasmin, α2-antiplasmin, is a major determinant of the effectiveness of plasminogen activators. α2-Antiplasmin is present in plasma both as a soluble protein and as a protein covalently linked to fibrin by amide bonds (mediated by factor XIIIa). Reed et al14 have shown that an antibody that selectively inhibits α2-antiplasmin can enhance the activity of streptokinase, urokinase, and rt-PA 30- to 80-fold in vitro without increasing fibrinogen depletion. Using a human thrombus in a rabbit model, the authors demonstrated an enhancement of approximately threefold in the efficacy of rt-PA in vivo.7 Because the covalent linkage between α2-antiplasmin and fibrin defines an immunologic epitope, it can be used to differentiate circulating α2-antiplasmin from fibrin-bound α2-antiplasmin. An antibody specific for this epitope would permit selective inhibition of fibrin-bound α2-antiplasmin, thereby promoting enhanced plasmin activity at the thrombus without promoting a systemic lytic state. This epitope may also provide a unique targeting site for antibody-linked plasminogen activators.

An alternative method of targeting uses the principle of the bifunctional antibody: antibodies of two different specificities are coupled by cross-linking15,16 or somatic cell fusion.17,18 One antibody combining site is directed at an epitope in the thrombus, such as fibrin, the other at a plasminogen activator (scu-PA or t-PA) epitope distant from the catalytic site (so as not to inactivate the enzyme). Using a cell fusion product containing antibody combining sites specific for fibrin and t-PA, Branscomb et al18 showed an approximately 10-fold enhancement in the activity of rt-PA in the rabbit jugular vein model.

In their report in this issue, Collet et al15 used a cross-linking reagent to join the antibody to the plasminogen activator. A more sophisticated alternative, probably better suited for large-scale production and ultimate clinical use, is the biosynthesis of a single protein containing both an antibody combining site and a plasminogen activator catalytic unit. With recombinant DNA technology, two different genes can be linked in a manner that results in the expression of a single protein containing sequences encoded by both genes. Using this approach, Schnee et al19 generated a transfected (a hybridoma transfected by bacterial plasmid DNA) that secreted a single protein containing the Fab portion of an antifibrin antibody and the catalytic portion of t-PA. This hybrid bound fibrin as well as the parent antibody did and also activated plasminogen. More recently, Runge et al20 generated a similar fusion protein that contained an antifibrin antigen combining site and the catalytic unit of scu-PA. This protein was approximately 20 times more potent than scu-PA in pilot studies in the rabbit jugular vein thrombosis model.

The application of recombinant DNA technology affords two other abilities: to control antigenicity and to regulate pharmacokinetics. The antifibrin–scu-PA conjugate described by Collet et al5 consists of a mouse antibody coupled to a human plasminogen activator by an organic molecule. Both the mouse antibody and the cross-link can act as antigens. In a fusion protein, the cross-link is eliminated because both proteins are in contiguous peptide linkage, and methods are available with which to substitute human immunoglobulin sequences for most of the mouse structure.20 A more direct although as yet untested solution to the antigenicity problem is to clone a desired human antibody directly from the pool of genetic material in human peripheral blood lymphocytes.21 It is also possible to selectively alter the various structural elements that determine the plasma clearance of plasminogen activators without impairing their catalytic efficiency. These include elements that interact with plasminogen activator inhibitors22 and glycosylation sites.23 After the optimal epitope in one (or several) of the components of the clot has been selected, as well as the most effective plasminogen activator catalytic site, a novel protein that possesses the desired properties can be constructed.

Because the presently available plasminogen activators are quite effective and major complications resulting from their use are relatively infrequent, clinical studies aimed at proving the advantage of a new
plasminogen activator will have to be very large. Any required effort would be well justified because of the frequency of life-threatening thrombosis in clinical practice and the consequent impact of an improved therapy. Indeed, it is precisely this field that has pioneered the extensive multicenter trials that have a statistical power adequate to answer the question of whether plasminogen activators can be improved.

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_Circulation_. 1990;82:1874-1876
doi: 10.1161/01.CIR.82.5.1874

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1990 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/82/5/1874.citation