SPECIAL LECTURE

ANTIBODY-TARGETED THROMBOLYTIC AGENTS

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Plasminogen activator therapy for acute myocardial infarction has become standard medical practice. Bleeding complications, however, limit the utility of the currently available agents. This article reviews how the tools of immunology, molecular biology and protein engineering are being used to develop safer and more effective plasminogen activators.

The occlusion of a cerebral or coronary artery by a blood clot accounts for the majority of deaths in industrialized countries and is responsible for significant incapacitation and morbidity! The immediate cause of occlusion is a defect in the vessel wall, usually atherosclerotic, but it is the formation of a thrombus on this defect2 that almost invariably results in the final interruption of blood flow. Perhaps prevention of atherosclerosis will have the most important effect on this process; yet for many in whom prevention is too late, dissolution of the thrombus with plasminogen activator therapy is an attractive method of restoring blood flow.

The activation of plasminogen results in the formation of plasmin, a proteolytic enzyme that degrades fibrin, the principal component of the structural lattice of a thrombus. In the past several years plasminogen activators have been shown to be effective therapeutic agents in coronary artery thrombosis, improving overall survival3–9 while limiting the degree of damage to the heart7,8,10. Unfortunately, because plasmin modifies platelet function11,12 and degrades circulating fibrinogen and clotting factors V and VIII13 as well as thrombus-bound fibrin, plasminogen activator therapy also carries the risk of hemorrhage. When bleeding occurs within the central nervous system, it usually results in grave neurological deficiency and often in death.14 Must the risk of bleeding accompany any effort to bring about thrombolyis? Perhaps the ability to selectively rather than systemically generate plasmin would improve the safety of plasminogen activator therapy.

Our review proceeds from the assumption that the ideal thrombolytic agent should be capable of attacking the components of a thrombus while sparing the circulating clotting proteins and platelets. Those components that have been modified by the clotting process offer the most easily defined targets toward which to direct a clot-selective agent. Fibrin, which differs in its covalent structure from its circulating precursor fibrinogen, is one obvious choice. Alternative targets include platelets altered during the clot-

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ting process, epitopes comprising the junction between platelet receptors and the proteins that bind to them [such as von Willebrand's factor, fibrinogen, and fibronectin\(^{15-17}\)], and the covalent link between \(\alpha_2\)-antiplasmin and fibrin. Here we restrict our attention to the fibrin selectivity of plasminogen activators, both as an intrinsic property of certain naturally occurring proteins and as a property that, through the methods of protein engineering, can be enhanced in thrombolytic agents.

**Clinical Use of Plasminogen Activators**

Although plasminogen activators have been used to treat both arterial and venous thrombosis at many sites within the body, their most dramatic application has been in the therapy of coronary occlusion. Streptokinase, a bacterial protein, and urokinase, a human enzyme initially purified from urine, constitute the first generation of plasminogen activators. When administered within 4h of coronary occlusion, streptokinase has been shown to reduce mortality after myocardial infarction by 23 to 81 percent in a number of randomized trials\(^6,7,9,18\). However, the use of this agent is invariably accompanied by a marked depletion of fibrinogen caused by the generation of excess plasmin. Unchecked, the systemic release of plasmin is capable of paralyzing the clotting process, causing what is known as a “lytic state”\(^9\). Results obtained with urokinase have been similar in smaller-scale clinical trials\(^20,21\).

More recently a second generation of plasminogen activators has become available: tissue-type plasminogen activator (tPA) and single-chain urokinase-like plasminogen activator (scuPA). Unlike streptokinase and urokinase, tPA and scuPA exhibit fibrin-selective plasminogen activation. Although there is now some controversy as to whether the fibrin-selective agents have any clinical advantage over the nonselective agents\(^22\).

*Fig. 1. Two-dimensional representation of the structures of (A) tissue-type plasminogen activator (tPA), (B) single-chain urokinase-like plasminogen activator (scuPA), and (C) plasminogen. (A) The A chain of tPA is NH\(_2\)-terminal and the B chain COOH-terminal to the plasmin cleavage site, P1, between residues 275 and 276. The functional domains are designated F, finger; E, epidermal growth factor-like; K, kringle; and C, catalytic. D indicates the limits of the individual functional domains deleted by Gething et al.\(^23\). The dashed lines represent intrachain disulfide bonds, the zigzags N-linked oligosaccharides. (B) The A chain of scuPA is NH\(_2\)-terminal and the B chain COOH-terminal to the plasmin cleavage site between residues 158 and 159, H, that results in high molecular weight two-chain urokinase. A second plasmin cleavage site, L, results in low molecular weight two-chain urokinase. Cleavage at LM gives rise to low molecular weight scuPA. Other symbols as in (A). (C) Cleavage at P1, between residues 560 and 561, activates plasmin to plasminogen. [Adapted from\(^5\).]*

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there is little disagreement that a general disruption of the clotting system is an undesirable state.

The selectivity of tPA derives from the presence of a fibrin binding site on the molecule. tPA binds fibrin with a $K_d$ of 0.16 $\mu$M; when bound, its $K_m$ for plasminogen activation decreases from 83 $\mu$M to 0.18 $\mu$M and its $K_{cat}$ increases from 0.07 to 0.28 sec$^{-1}$, resulting in an increase in catalytic efficiency of approximately 1000-fold. Although scuPA probably does not bind directly to fibrin (see below), it activates fibrin-bound plasminogen much more readily than plasma plasminogen. Its fibrin selectivity is comparable to that of tPA.$^{23}$

tPA and scuPA are also considered native plasminogen activators because endothelial (and other) cells secrete them into the circulation. Initial studies of tPA and scuPA were conducted on proteins purified from cultured cell lines: melanoma cell line RPMI 7932 for tPA, and transformed human kidney cells for scuPA. Both agents have subsequently been produced by recombinant DNA methods.$^{24,25}$

The cleavage of native, single-chain tPA by plasmin between amino acids Arg$^{275}$ and Ile$^{276}$ produces two-chain tPA, which is similar in fibrin selectivity and catalytic activity to its single-chain precursor. scuPA is cleaved by plasmin between amino acids Lys$^{158}$ and Ile$^{159}$. The resulting high molecular weight two-chain urokinase has the catalytic activity of scuPA, but does not have the fibrin selectivity and resistance to plasminogen activator inhibitor I of its single-chain precursor. Low molecular weight two-chain urokinase is the first generation form (Fig. 1). The full length, high molecular weight form of scuPA is the native plasminogen activator and is the form that has been studied clinically as a second generation plasminogen activator.

tPA has been shown to reduce mortality by 23 to 36 percent and to result in a depletion of fibrinogen less severe than that caused by streptokinase.$^{26,27}$ This mitigation of fibrinogen depletion is probably because of tPA's ability to bind fibrin, which would promote the activation of plasminogen in close proximity to a thrombus. Nevertheless, at doses required for the prompt lysis of coronary thrombi, the administration of tPA can also result in hemorrhage$^{26,27}$ accompanied by depletion of both fibrinogen and plasminogen.$^{26}$ Although fibrinogen and plasminogen losses are generally less after tPA administration than after streptokinase adminis-

tration, it should be noted that measurement of a few selected proteins may not accurately reflect loss of other, more important elements of the clotting system. Clinical experience with scuPA is still limited, although it appears to be an effective thrombolytic agent$^{28}$; as with tPA the administration of scuPA is also associated with hemorrhage.

Almost as frequently observed in clinical trials has been the tendency for arteries recanalized by tPA to reocclude$^{29,30}$ with subsequent loss of the myocardium that had originally been in jeopardy. Not surprisingly, streptokinase, which, as discussed earlier, severely depletes circulating clotting proteins, is associated with a lesser incidence of reocclusion than is tPA.$^{27,29}$ Streptokinase also reduces blood viscosity, probably because of marked fibrinogen depletion$^{31}$; whether this effect reduces the incidence of reocclusion is not clear. Preliminary evidence suggests that reocclusion may not be so frequent with scuPA as with tPA$^{32}$ but a direct comparison has yet to be reported.

Thus despite their fibrin selectivity relative to the first generation of plasminogen activators, the second generation is also beset by significant limitations. The design of a third generation of plasminogen activators composed of more selective molecules will require a detailed understanding of structure-function relationships of plasminogen activators.

Structure and Function of Native Plasminogen Activators

tPA and scuPA are both multidomain proteins. An examination of the function of the individual domains, made either by isolating them after selective proteolytic cleavage or by deleting the DNA segments that encode for their synthesis, may provide the insights necessary to overcome some of the disadvantages inherent in the first and second generation agents.

tPA and scuPA have highly homologous COOH-terminal segments that are responsible for their catalytic activity. In tPA the NH$_2$-terminal segment effects fibrin binding, whereas in scuPA the function of the NH$_2$-terminal region has not been determined. In scuPA the NH$_2$-terminal (or A) chain is lighter than the COOH-terminal (B) chain; in tPA the A chain is the heavier (Fig. 1, A & B).

Structural information is far more complete for tPA than for scuPA. Five domains exist in tPA, each of which is encoded by a single
exon or group of exons33 (Fig. 1A). The heavy chain (NH2 terminus) of tPA contains 4 domains: a "finger" domain similar to that of fibronectin,34 an epidermal growth factor (EGF)-like domain homologous to that of urokinase, protein C, and coagulation factors IX and X35 and 2 "kringle" domains joined by intradomain disulfide bonds similar to those found in plasminogen, scuPA, and prothrombin. The light chain (COOH terminus) contains the fifth domain, which is homologous to the catalytic regions of other serine proteases.

Gething et al33 have recently described the properties of 5 deletion mutants from single-chain tPA. The mutants were constructed by modifying intron/exon junctions so that the exon encoding each of the 5 tPA domains could be precisely deleted. The finger or EGF-like domain (or both) appear to contribute to the high-affinity interaction between tPA and fibrin. Interestingly, mutants lacking only the finger domain display an increased affinity for plasminogen. The absence of the EGF-like domain does not of itself cause a difference in in vitro properties, although the effect of its absence has not been examined in vivo. The 2 kringle regions appear to be highly homologous and to have equivalent abilities to mediate the stimulation of catalytic activity by fibrin, as evidenced by the fact that deletion mutants lacking both kringles do not bind to fibrin and do not show enhancement of catalytic activity in the presence of fibrin33. Plasminogen activator inhibitor I binds to and inhibits the catalytic segment of tPA. As expected, deletion of all 4 domains composing the fibrin binding segment does not diminish interaction with plasminogen activator inhibitor I. These results, together with those of van Zonneveld et al36 MacDonald et al37 and Larsen et al38 provide convincing evidence that the 5 structural domains of tPA have different and autonomous functions.

The light chain (NH2 terminus) of scuPA contains, in addition to an EGF-like domain, a single kringle region that shows considerable homology with the kringles of tPA (despite the fact that scuPA does not appear to bind fibrin).39–41 (Fig. 1B). One property that differentiates scuPA from tPA is scuPA’s resistance to irreversible inhibition by plasminogen activator inhibitor I (as well as to other plasminogen activator inhibitors). For this reason, unlike tPA, scuPA is stable in human plasma for extended periods. (Plasminogen activator inhibitor I binds reversibly to scuPA: when scuPA forms a ternary complex with fibrin and plasminogen, plasminogen activator inhibitor I is displaced.) It is not until after plasmin cleaves scuPA between residues Lys158 and Ile159 to form high molecular weight two-chain urokinase that the catalytic site becomes susceptible to irreversible inhibition. Low molecular weight two-chain urokinase derives from subsequent cleavage of the Lys135–Lys136 peptide bond. It too is readily inhibited by plasminogen activator inhibitor I.

**Antibody-Targeted Fibrinolysis**

In light of a number of published experiments42–46 it would seem that the shuffling of domains from existing components of the fibrinolytic system, whether by chemical or recombinant DNA methods, cannot significantly increase fibrin selectivity beyond the point attained by the parent plasminogen activators. An alternative approach to the problem is to use a fibrin-specific antibody in conjunction with a plasminogen activator catalytic site, to achieve an affinity and a selectivity greater than those afforded by the fibrin binding domain of plasminogen or tPA.

The reagent critical to this approach is an antibody that binds to fibrin with high affinity but does not cross-react with fibrinogen. Thrombin acts on fibrinogen to cleave peptides from the NH2 termini of the fibrinogen Aα and Bβ chains, leading to the noncovalent polymerization of fibrin. The newly revealed six-amino acid sequence at the NH2 terminus of the fibrin β chain is an epitope that is not available on fibrinogen. We have developed monoclonal antibodies to a synthetic peptide containing this sequence47 which bind to fibrin with high affinity (0.77 nM) but do not bind to fibrinogen. In contrast, the binding constant of tPA for fibrin is 0.16 μM. We subsequently harnessed the fibrin selectivity of these antibodies and their fragments to the catalytic activity of plasminogen activators—first as plasminogen activator-antifibrin antibody conjugates, and, more recently, as bifunctional antibodies with specificities for both a plasminogen activator and fibrin.

**Plasminogen Activator-Antifibrin Antibody Conjugates**

Antifibrin antibody 59D8 (or its Fab) was chemically conjugated to urokinase and tPA by means of the disulfide cross-linking reagent N-succinimidyl 3-(2-pyridyldithio) propionate48,49

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The resulting urokinase-59D8 and tPA-59D8 molecules were, respectively, 100-fold more potent than urokinase and 10-fold more potent than tPA in an in vitro fibrinolytic assay. To determine whether this increased efficacy was physiologically relevant, we compared tPA-59D8 with tPA alone in a human plasma clot assay and in the rabbit jugular vein model (in vivo). tPA-59D8 was 3.2-fold more potent than tPA alone in human plasma and 3.0- to 9.6-fold more potent than tPA alone in vivo (over a range of tPA concentrations; the greatest benefit was observed at the lowest tPA concentrations).

Both in vivo and in vitro, this increased fibrinolytic potency was accompanied by a decrease in the consumption of plasminogen, α2-antiplasmin, and fibrinogen, indicating that the increase in potency is the result of an increase in selectivity. High molecular weight scuPA chemically cross-linked to antibody 59D8 appears to effect similar increases in fibrinolytic potency.

Opportunities to chemically link plasminogen activator-antifibrin antibody conjugates are limited to the coupling of available proteolytic fragments such as Fab and the B chain of urokinase or tPA. To permit a detailed search for the optimal structure of a molecule containing both a fibrin-specific antibody binding site and a plasminogen activator catalytic site, our laboratory applied recombinant DNA methods. These techniques allow the flexibility of combining a minimal antigen binding structure [the 25-kD variable domain antibody molecule (Fv)] with a minimal catalytic domain in a variety of arrangements and at various molecular distances. Two recombinant activator-antibody constructs have been tested thus far, each containing the rearranged 59D8 heavy-chain gene. In the first construct the variable exon of the 59D8 heavy-chain gene was combined with the CH1 and hinge exons of mouse immunoglobulin γ2b and the cDNA coding for the B chain of tPA to form the expression vector pSVD8tB52. The plasmid was then transfected into a clone of hybridoma 59D8 that had lost the ability to produce heavy chain. Clones resistant to mycophenolic acid (that is, containing the Escherichia coli guanine phosphoribosyltransferase gene of pSVD8tB) secreted a protein of 170 to 180 kD that was recognized by both fibrin- and tPA-specific antibodies. The molecule contained 2 unmodified antibody light chains of 25 kD each, and 2 heavy chains of 65 kD each (Fig. 2). The heavy chains proved to be fusion peptides, extending from residues 1 to 236 of the antibody's heavy chain and from residues 275 to 527 of tPA (the amino acids corresponding to the B chain).

The tPA-59D8 recombinant construct bound fibrin with an affinity equal to that of the parent antibody. Its amidolytic activity, measured as the ability to cleave chromogenic substrate S2288 (H-D-isoleucyl-L-prolyl-L-arginine-p-nitroanilide dihydrochloride), was 70 percent that of single-chain tPA and its activity as a plasminogen activator, measured in highly purified assays in the absence of fibrin, was equal to that of tPA (when corrected for the loss in amidolytic activity). Surprisingly, however, the chimeric molecule was ineffective in lysing a plasma clot. This was in contrast to results our laboratory had obtained earlier with a chemical conjugate of the B chain of tPA and 59D8 Fab, which had proved as potent as tPA in fibrinolysis. The presence in the recombinant construct of Gly instead of the expected Arg would have prevented the plasmin cleavage at this position that normally results in the two-chain form of tPA. We infer that the consequent absence of a free NH2 terminus on the B chain of tPA prevented the fibrin-mediated enhancement of catalysis that occurs with native tPA.

The second construct shared with the first
the 59D8 variable region exon and the mouse immunoglobulin γ2b CH1 constant region and hinge exons, but also contained a γ2b CH2 exon and the exons coding for the entire sequence of low molecular weight scuPA (in place of the sequence coding for the B chain of tPA)53. Transfection into 59D8 hybridoma cells that had been selected for the inability to produce heavy chain resulted in a secreted protein that is a monomer containing both immunoglobulin and scuPA epitopes. This scuPA-59D8 chimera differs from the tPA-59D8 protein by the fact that it contains a plasmin-sensitive bond (in this case the Lys135-Lys136 bond in scuPA). In contrast to recombinant tPA-59D8, recombinant scuPA-59D8 is a highly effective fibrinolytic agent, demonstrating an increase in potency of approximately tenfold relative to scuPA. More recent antifibrin antibody constructs, containing a larger segment of tPA in which the plasmin cleavage site as well as 2 kringle are conserved, also show increased activity relative to native tPA in an in vitro fibrinolytic assay. Although a great deal of exploration is still required before the optimal configuration of a plasminogen activator-antifibrin antibody chimera can be determined, it does appear possible to endow plasminogen activators with enhanced fibrin selectivity through the incorporation of a fibrin-specific antibody binding site.

Bispecific Antibodies

A bispecific antibody that recognizes epitopes on both fibrin and a plasminogen activator should be capable of increasing the effective concentration of the plasminogen activator in the proximity of a fibrin deposit. We have synthesized an antiantiactivator-antifibrin antibody by chemically cross-linking fibrin-specific antibody 59D8 to tPA-specific antibody TCL8 (Kd of 5 × 10⁻⁹ M). The resulting conjugate increased the potency of tPA by fivefold and consumed less fibrinogen and α2-antiplasmin in vitro and in vivo (in the rabbit jugular vein model)54. Similar results were obtained in vitro with a bispecific antibody recognizing fibrin and urokinase55.

An alternative and probably more effective method of synthesizing bispecific antibodies is the hybrid-hybridoma technique56. We have subjected clones from a hybridoma producing thymidine kinase-deficient 59D8 and a hybridoma producing hypoxanthine guanine phosphoribosyltransferase-deficient TCL8 to somatic cell fusion in ethylene glycol, and selected the surviving clones in hypoxanthine aminopterin thymidine medium. The antibodies secreted by the hybrid hybridomas proved to bind both fibrin and tPA. In an in vitro fibrinolysis assay, the bispecific, hybrid-hybridoma antibodies increased the potency of tPA by 11-fold57. In more recent studies in vivo, in the rabbit jugular vein model, the enhanced potency of these hybrid hybridomas was also demonstrated. Because of their uniformity, stability, and ease of production, hybrid-hybridoma products are likely to be better candidates for in vivo study than the chemically cross-linked conjugates.

Conclusion

Plasminogen activators are composed of independent functional domains that provide an exceptional opportunity for protein engineering aimed at improving the properties of the natural molecules. Thus far, efforts have focused on the issues of enhanced fibrin selectivity and extended in vivo half-life. Experiments in which functional domains of plasminogen and urokinase, plasminogen and tPA, or tPA and low molecular weight scuPA are joined have provided significant insight into the function of the constitutent plasminogen activators, but the hybrids have not had greatly enhanced fibrin selectivity. However, the combination of a high-affinity fibrin-specific antibody binding site and a tPA or scuPA catalytic site appears to increase both the potency and selectivity of the parent plasminogen activator.

A plasminogen activator with enhanced fibrin selectivity cannot differentiate between a thrombus that threatens the heart and one that prevents exsanguination in a gastric artery. For this reason, bleeding will be an occasional consequence of even the most fibrin-selective plasminogen activator. Yet because a fibrin-selective plasminogen activator would restrict the action of plasmin to the site of a thrombus, the extent of the activator's effect would be limited to the time it takes for the activator to be eliminated. Thereafter the hemostatic system would return to normal. This effect would be in contrast to that of streptokinase, which necessitates the synthesis and secretion of new clotting proteins before normal hemostasis is restored.

In engineering the ideal plasminogen activator, one must determine the optimal selectivity as well as the precisely appropriate duration of action. The potential exists for enhancing selectivity beyond localization of the fibrinolytic
process to the site of a thrombus. It may be possible to differentiate between recently formed and older thrombi on the basis of relative degrees of interchain cross-linking. On might hope that a coronary thrombus that presents as acute ischemic pain would be of more recent origin than a silent fibrin plug that holds back potential bleeding. There is also a growing understanding of the determinants that govern the clearance of a protein from the circulation. By appropriate engineering, it may be possible to impart a circulatory half-life that is consistent with the therapeutic goals of clot dissolution and prevention of reocclusion, yet avoids the risk of an excessively long impairment of the hemostatic system. The work reviewed in this article is but the background and the beginnings of the search for the ideal thrombolytic agent.

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