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TISSUE PLASMINOGEN ACTIVATOR: The Biochemistry and Pharmacology of Variants Produced by Mutagenesis

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INTRODUCTION

Thrombi, whatever their locations or etiologies, are to some extent composed of and held together by fibrin. Fibrin is formed by the action of thrombin on soluble fibrinogen and is the end result of the complex series of reactions in the coagulation cascade. Under normal circumstances the deposition of fibrin occurs at sites of blood vessel injury to stem the loss of blood from the vessel, and it is essential that the fibrin deposit be maintained until tissue repair has been initiated. Fibrinolysis comes into play as the mechanism by which fibrin is removed from the system. Regulation of the initiation of thrombolysis following wound repair is poorly understood, but it is clear that plasminogen activators are capable of converting plasminogen to plasmin, which degrades the fibrin matrix to soluble, readily cleared products. Under unusual circumstances, such as in myocardial infarction, a blood clot is deposited in such a way that blood flow through the vessel is significantly reduced. The damage initiating this misplaced coagulation event is the subject of considerable research; however, in the absence of ways to prevent such occurrences, it is essential that the thrombi be removed promptly. During myocardial infarc-

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tion, the intrinsic fibrinolytic response is apparently insufficient to remove the fibrin deposit before a lack of blood supply has caused damage to the surrounding tissue. An increased understanding of the mechanisms of clot formation and dissolution has led to the development of several different fibrinolytic agents for the treatment of such conditions. Streptokinase (SK), urokinase (u-PA), and tissue plasminogen activator (t-PA) are all capable of converting plasminogen to plasmin. These fibrinolytic agents, all of which have been used successfully in thrombolytic therapy, differ somewhat in their mechanisms of action. SK and u-PA activate circulating plasminogen and rely on circulating plasmin to associate with fibrin, whereas t-PA is thought to prefer clot-associated plasminogen as the substrate (1) and is thought to produce less systemic consumption of plasminogen, α_2-antiplasmin, and fibrinogen than the other two agents do (2–6).

t-PA was demonstrated to be a useful thrombolytic agent by Collen and coworkers (1, 7, 8), using material that had been purified from the spent medium of cultured Bowes melanoma cells (7, 9). Up to that time, the best natural sources of human t-PA had been thiocyanate perfusates of human cadavers (10, 11) or extracts of uterine tissue (12). Techniques of molecular biology that became available in the early 1980s were used to clone and express the complementary DNA (cDNA) encoding human t-PA (13), and sufficient quantities of recombinant t-PA (rt-PA) for use in clinical trials were subsequently produced (14, 15). rt-PA was approved in 1987 for treatment of acute myocardial infarction in the United States under the trade name Activase®. It was already apparent, however, that although t-PA had advantages over u-PA and SK, it was considered amenable to improvement. Its action is slower than might be desirable, with a time to lysis of about 60–90 min (5); its fibrin specificity is not absolute (some patients experience significant decreases in the levels of circulating plasminogen, α_2-antiplasmin, and fibrinogen) (6, 16); the effect is not necessarily long-lasting (a significant number of reocclusion events have been noted) (17, 18); bleeding is considered to be a potential side effect of concern (6); and it is metabolized rather quickly, with an in vivo half-life in humans of about 4 min (19). Not surprisingly, the techniques of genetic engineering that allowed recombinant t-PA to be produced in the first place have been applied to altering its properties. In this review, we discuss the pharmacology of various second-generation t-PA molecules, as well as review the structural and functional characteristics that are the primary targets of the directed mutagenesis efforts involving t-PA at present. We also attempt to correlate the known in vitro properties of t-PA and its variant forms with their pharmacological effects.

Structure of t-PA

Both t-PA and u-PA are plasma proteins consisting of a serine protease domain at the carboxyterminal end and several other domains typical of

plasma proteases at the amino-terminal end (20) (Figure 1). Homology analysis (13, 21) indicates that mature t-PA has a total of five domains of four different types: a fibronectin-type finger region of about 44 amino acid residues, a growth factor-type domain of about 40 residues, two kringle domains of 81 residues each, and a protease region of about 260 residues. Linker regions bring the total to 527 residues for the mature protein (13), although three additional residues (Gly-Ala-Arg) are occasionally found at the amino terminus (22–25). This additional tripeptide is generally thought to be the result of incomplete precursor processing, and it is not known to impart functionality.

The human t-PA gene is divided into exons coding separately for the various domains. The signal peptide, propeptide, finger, and growth factor regions are encoded by individual exons, whereas the kringles and the protease domain are encoded by multiple exons (26). The mRNA for t-PA codes for a precursor of 35 additional residues at the amino terminus of the protein (of which the above-mentioned tripeptide Gly-Ala-Arg is the carboxy-terminal part). The first 20 residues constitute a typical hydrophobic signal sequence and are followed by a positively charged decapeptide that may be a processing signal. The hydrophobic region of the precursor peptide contains three cysteines (13).

Mature t-PA contains 35 cysteines. Homology with other plasma proteases allows 34 of the cysteines to be tentatively assigned to 17 disulfide bonds, as indicated in Figure 1A (13, 21). No inconsistencies with these assignments have been reported (although few of the disulfides have been confirmed experimentally). The most likely site for the unpaired cysteine in t-PA is at position 83, in the growth factor domain. It is possible that the adjacent cysteine 84 is the unpaired one, but the region occupied by these residues is homologous with other epidermal growth factor–like domains (21). Most of these have a disulfide corresponding to positions 75 and 84, and the assignment was based on this homology leaving cysteine 83 unpaired.

The t-PA protease domain is about 35–40% homologous with prototypic serine proteases such as bovine trypsin and chymotrypsin. The regions of highest homology are the residues including and flanking those of the active site: H322 and S478 (corresponding to H57 and S195, respectively, on serine proteases). The lowest homology is in regions of t-PA around residues 298, 330, and 470, for which there are no corresponding residues in the prototypes. One region of peculiar homology is the amino terminus of the protease domain (residues 276–279). The prototype has two hydrophobic residues such as Ile or Val followed by two β-turn residues such as Gly or Asn. t-PA has the sequence IKGG, with the lysine residue being a rarity at that position in serine proteases.

The amino-terminal "finger" region is about 25–30% homologous with corresponding regions in fibronectin, and the growth factor region is about

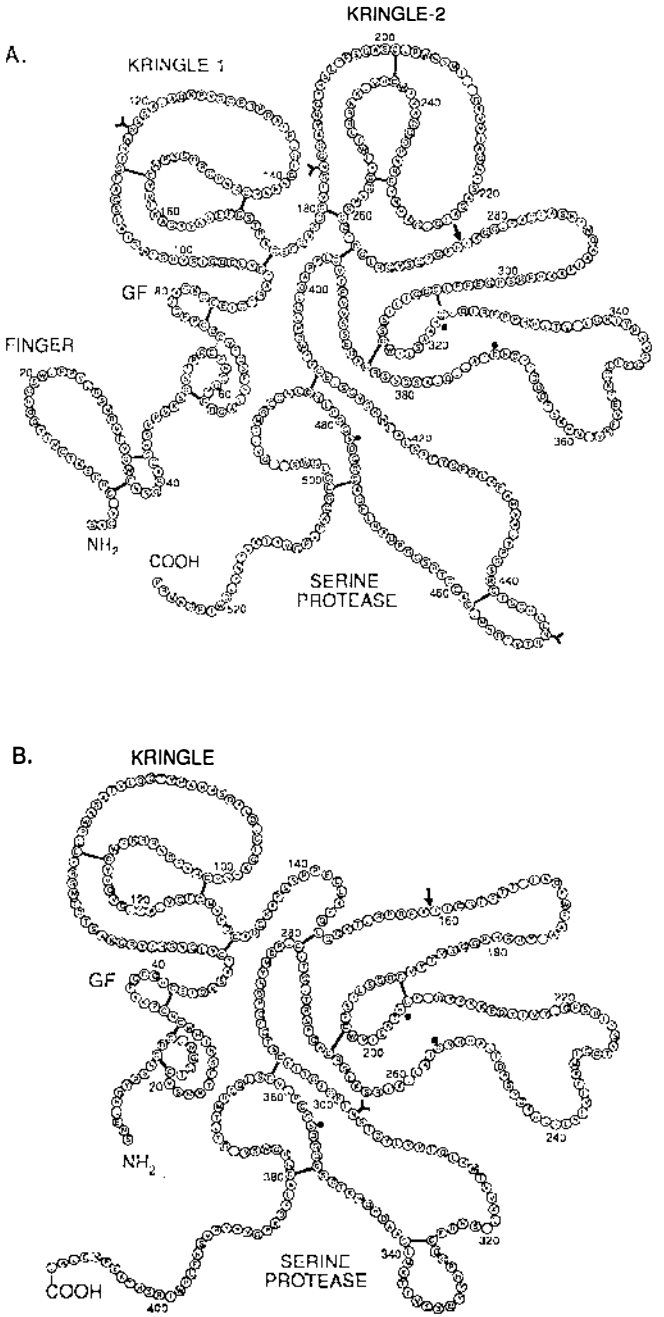

Figure 1 (*A*) Structure of t-PA. (*B*) Structure of and u-PA. Single-letter codes are used to indicate the amino acid residue present at each site in the wild-type proteins. Symbols: ●, active-site residue; Y, glycosylation site; →, "activation" site where hydrolysis causes the one- to two-chain conversion.

25% homologous with a region of human epidermal growth factor (although this homology is almost exclusively confined to cysteine residues). The kringles are 30–50% homologous with kringle structures on other plasma proteins. The highest homology is with the u-PA kringle (about 50% homologous with both t-PA kringles), but both t-PA kringles unexpectedly have about 40% of their residues in common with regions of the functionally unrelated apolipoprotein(a) (27).

No crystallographic studies have been published on full-length t-PA, and very little is known about the higher-order structure of the protein. Although the general structures of kringles (28, 29) and of serine proteases are reasonably well understood, neither the general structures of the other domains nor the exact structures of any t-PA domains are known at present. Furthermore, virtually nothing is known of the relative orientations of the t-PA domains; modeling a structure of the entire protein is therefore highly speculative. It is clear, however, that the interdomain interactions of t-PA are important to its unique properties. The fact that t-PA has very little activity toward plasminogen in the absence of fibrin (or a fibrinogen-derived stimulator) suggests that the enzyme is normally in an inhibited state and that some structure on fibrin is able to interact with t-PA to overcome that inhibition. When found in other proteins, two of the domain types on t-PA (fingers and kringles) are known to interact with fibrin, and it is tempting to think that these domains are in some way responsible for the inhibition, and the release of that inhibition.

Oligosaccharide Structures on t-PA

There are four consensus N-linked glycosylation sites in the mature t-PA sequence, and three of these (at positions 117, 184, and 448) are known to be glycosylated under some circumstances (30, 31). The fourth, at position 218, has not been found to become glycosylated by mammalian cells (23, 31, 32), probably owing to the presence of the neighboring proline residue at position 219. Both melanoma-derived and recombinant CHO cell–derived t-PA exist in two readily distinguishable subtypes, which are referred to as types I and II (30, 32, 33). Type I is glycosylated at positions 117, 184, and 448, whereas type II is glycosylated only at positions 117 and 448. The carbohydrates at positions 184 and 448 are of the complex type, whereas position 117 is occupied by a high-mannose-type carbohydrate (31, 32, 34). Type I and II t-PA have been shown to differ in their specific activities (24) and in their in vitro affinities for fibrin (N. Paoni & B. Keyt, personal communication), with type II being slightly higher in both cases. Type I, by virtue of its additional carbohydrate, is somewhat more soluble (T. Nguyen, personal communication).

The most comprehensive analysis of the carbohydrate structures on recombinant CHO cell–derived t-PA was carried out by Spellman et al (35), who

showed that at least 17 different Asn-linked carbohydrate structures could be detected on the protein. These ranged from the high-mannose structures at position 117 to di-, tri-, and tetra-antennary *N*-acetyllactosamine-type structures at positions 184 and 448. The presence or absence of an oligosaccharide at position 184 (as between type I and II t-PA) did not influence the compositions of the oligosaccharides at the other two positions. This observation is noteworthy within the context that alterations in the peptide sequence can affect the oligosaccharide compositions, as discussed below.

Functions of t-PA

The accepted activity of t-PA is to cleave the R561–V562 peptide bond of plasminogen, converting it to plasmin. The cleavage site is located on a 9-residue disulfide-bonded loop with the sequence CPG**R**VVGGC. This loop, consisting mainly of β-turn residues surrounding the cleavage site, is probably relatively rigid; this may be a determinant of the substrate specificity of t-PA and u-PA. Interestingly, the site of t-PA and u-PA cleavage on PAI-1 (see below), between residues R346 and M347 (36), bears little resemblance to the plasminogen loop.

One of the unusual functions of t-PA is its ability to act as a protease in the one-chain form. By analogy with serine protease zymogens, including u-PA, one would predict an inactive single-chain t-PA, which would be activated by hydrolysis of a bond between residues R275 and I276 which would cause a conformational change involving the new amino terminus and results in the ordering of active-site residues. t-PA can be isolated in either the one- or two-chain form from tissue culture supernatants (7) and can be readily converted from the one- to the two-chain form by the action of plasmin (32, 37). One-chain t-PA is certainly capable of hydrolyzing small-peptide substrates such as D-Ile-Pro-Arg paranitroanilide, although the Michaelis constant (K_m) is about threefold higher than that for the two-chain enzyme. Because the one- to two-chain conversion is accomplished by plasmin (the product of the action of t-PA upon its substrate) and t-PA is so readily converted to the two-chain form during a reaction, it was difficult to establish that one-chain t-PA is active against plasminogen (38). It is now well accepted (as described below) that one-chain t-PA is active against plasminogen (38, 39) and that with the exceptions that one-chain t-PA binds fibrin somewhat more avidly (40) and inhibitors less avidly (41–44) than two-chain t-PA does, most of the properties of the two forms are similar.

Inhibition of t-PA

As a serine protease, t-PA is susceptible to irreversible inhibition by some general inhibitors, such as diisopropylfluorophosphate (12, 33), and by some selective inhibitors containing arginine, such as Phe-Pro-Arg-chlorome-

thylketone (43–45). These agents are reactive against either one- or two-chain t-PA. The peptide inhibitor aprotinin, which does inhibit u-PA and plasmin (46), is ineffective against either form of the t-PA molecule. Several higher-molecular-weight plasma-derived inhibitors react with and inhibit t-PA. Relatively slow inhibitors, not considered physiologically important, are α_2-antiplasmin, C1-esterase inhibitor (33), and α_2-macroglobulin (47). Displaying moderate rates of inactivation are protease nexin and the placental inhibitor PAI-2 (48). A relatively fast-acting inhibitor of t-PA and two-chain u-PA (36), usually referred to as PAI-1, is present in plasma (49–52), endothelial cells (53, 54), and platelets (55–59). PAI-1 is a serpin-related protein (60–62) and, as such, is cleaved by t-PA or u-PA at the R346–M347 peptide bond (36), forming a stable complex. The activity of PAI-1 in vitro is unstable, possibly owing, in part, to its high susceptibility to oxidation (63). Its ordinary concentration in plasma (less than 20 ng ml^{-1}) (64, 65) would not be high enough to interfere significantly with t-PA therapy, but its concentration can be markedly increased during myocardial infarction (66) as well as during certain disease-related or physiological states (67–69). This inhibitor is most probably an important component of the intrinsic fibrinolytic system (48); as such, local concentrations of PAI-1 may be more important than its concentration in plasma. Variants of t-PA that are resistant to inhibition by PAI-1 are of considerable utility in testing this hypothesis.

CHALLENGES OF SECOND GENERATION t-PA RESEARCH

Target Changes

Two significant difficulties in trying to improve upon an approved pharmaceutical such as Activase are, first, determining what the clinical properties of an "improved" drug might be and, second, deciding what characteristics of the molecule should be altered to give the desired action. There is considerable disagreement about desired clinical traits. On one side, some investigators believe that a prolonged clearance rate would be desirable, by allowing lower doses of the drug and permitting administration by bolus rather than by infusion. Others counter that the short half-life of t-PA is actually an advantage, in that stopping the infusion would quickly terminate the action of the drug. More "fibrin specificity" and less plasminogen and fibrinogen depletion are often mentioned as being desirable. This goal is challenged by those who believe that the depletion of fibrinogen is helpful in avoiding significant problems with reocclusion. The ideal drug would be one that is effective immediately in all cases, has no side effects, and prevents reocclusion. It is questionable whether such a drug could be developed, especially as some of the side effects of fibrinolytic agents (e.g., intracranial

bleeding) may be the result of carrying out the intended function (fibrinolysis) in the wrong location.

Difficulty

It is unclear whether any of the currently used animal models are really predictive of the human situation, in which myocardial infarction is a sudden event triggered by the rupture of a plaque, itself the product of years of insult to the artery. Thus, the cellular milieu and clot architecture in animal models may be very different from the human situation. The lack of human clinical data on variants of t-PA makes it difficult to address issues concerning animal models at present.

Assuming that one does have an ideal in vitro model with which to assess functions that are known to provide improvements in t-PA with regard to thrombolytic therapy, there are still many potential pitfalls in research of this sort. These include the effect that various expression systems may have on the structure of the molecule, particularly the glycosylation; the effect of the mutation(s) on the secondary and tertiary structure of the protein; and the effect of mutation(s) on the quantification in the variant, which is most routinely measured by enzyme-linked immunosorbent assay (ELISA) with antibodies directed to wild-type t-PA.

VARIANT PROTEINS

Domain Deletions

Most of the first t-PA variants produced by site-directed mutagenesis were based on the homology of t-PA with other plasma proteins. Various groups have used this information to construct a series of deletion mutants in which individual domains of the molecule were removed. This approach is based on the assumption that each of the domains is an independently folding module, so that the removal of a complete domain might minimally affect the folding of the rest of the protein. These considerations necessarily avoid the issue of interdomain packing of the molecule, because the lack of physical data about t-PA has made it very difficult to assess the true effects of the deletion of a domain on the structures (and hence the functions) of other domains. Domains upstream of a deleted domain are clearly in nonstandard positions relative to the protease. Notwithstanding these ambiguities, however, the precise location of the domain deletion should be the subject of careful consideration. For the most part, the variants used by each investigating group are slightly different. Some groups used sequence homology to determine domain boundaries; other groups used the naturally occurring intron/exon boundaries (26) to determine the appropriate residues to delete. This latter approach appears to be the more appealing, since exon shuffling in vivo appears to have led to the

development of the t-PA gene through evolution (20). However, even when interdomain deletions were avoided and the deletions were made at sites of intron/exon junctions, little information is available to support the contention that a particular domain deletion variant has the "proper" conformation in the remaining portions of the molecule. Thus, results in which a deleted domain is deemed responsible for the loss of a function must be viewed with caution.

IN VITRO RESULTS The first deletion variant of t-PA was missing the finger domain (70). The cDNA coding for this variant was isolated from a library prepared from Detroit 562 cells and was found to contain the entire t-PA gene except for exon IV. The missing base pairs may have resulted from alternative splicing, but the possibility of an altered genomic structure in these cells cannot be excluded. The cDNA coding for the variant lacking the finger domain GK1K2P (Δ4–49 S50G)[1] was expressed in *Escherichia coli*. The authors investigated the interaction of this variant with fibrin by applying the purified variant to a fibrin Celite® column. They concluded that the fingerless form of t-PA did bind to fibrin. This result is in contrast with data obtained with more recent fingerless variants (all of which exhibit attenuated fibrin binding; see below).

The first series of domain deletions constructed by using DNA recombination techniques was published in 1986 by van Zonneveld et al (71–73). This group made a series of variants by mutating the A chain (amino-terminal half of the molecule) by using restriction endonucleases to delete one or more structural domains. All variants were expressed transiently in mouse Ltk^- cells and assayed without further purification. The proteins made were P (Δ−11–255), K1P (Δ−11–89 and Δ174–255), K2P (Δ−12–174), K1K2P (Δ−12–89), GK1K2P (Δ−12–45), FGP (Δ88–255), and FP (Δ55–255). In all of the variants missing the finger domain, a portion of the propeptide sequence has also been removed. The effect of these mutations on the processing of the proteins is not known. The authors found that the removal of the finger, growth factor, or kringle 2 domain almost completely abolished the ability of the plasminogen-activating activity of the variant to be stimu-

[1]Variants missing entire domains are listed by using single letters to list the domains still present in the variant molecule (F, finger domain; G, epidermal growth factor domain; K1, kringle 1 domain; K2, kringle 2 domain; P, protease domain) followed in parentheses by ΔX-Y, where X denotes the residue number of the first amino acid deleted and Y denotes the residue number of the last amino acid deleted. Point mutations and multiple mutations are listed with a single-letter amino acid code for the amino acid present in wild-type t-PA followed by the residue number followed by the single-letter amino acid code of the amino acid present in the new molecule. The numbering system is based on that of Pennica et al. (13). For example, GK1K2P (Δ4–49 S50G) indicates a variant containing all domains except the finger domain, where the amino acids from 4 to 49 inclusive are deleted and the serine normally found at position 50 in wild-type t-PA is replaced with a glycine.

lated by cyanogen bromide fragments of fibrinogen, with the removal of the kringle 2 domain having the largest effect. The removal of the kringle 1 domain had no effect on this type of "fibrin" stimulation. They also assessed fibrin binding (qualitatively) and found that the fibrin binding in variants missing kringle 2 was highly attenuated, whereas the binding in variants containing kringle 2 and missing a different domain was only slightly attenuated. The authors concluded that the ability of a variant to be stimulated by fibrin correlated with its ability to bind to fibrin (71). Using the same series of variants, van Zonneveld et al analyzed the interaction of the proteins with lysine-Sepharose® and determined the concentration of ϵ-aminocaproic acid required to compete for that binding (72). Although the FP and FGP variants bound to fibrin, neither bound to lysine to any significant extent (71). ϵ-Aminocaproic acid was shown to compete for the binding of the kringle 2–containing variants, suggesting that kringle 2 contains a lysine-binding site. The variants containing the finger domain appeared to bind to fibrin via a different mechanism. All of the deletion variants interacted with PAI-1, and the authors concluded that the protease region of t-PA governs that interaction (73). However, quantitative differences among the variants were not ruled out. This series of proteins was used to map epitopes of 23 monoclonal antibodies made against wild-type t-PA (74). Each monoclonal antibody could be mapped with the deletion variants, and the affinity of each monoclonal antibody for the native protein and for all variants that contained the relevant domain appeared similar. This series of experiments strongly supports the contention that each domain folds independently and that the variant proteins were not grossly misfolded.

Several other groups have also made series of domain deletion variants, although in most cases the exact amino acid sequence as well as the expression system varied. Verheijen et al (75) were the first to analyze purified proteins. Their series of variant proteins included GK1K2P (Δ5–44), K2P (Δ7–168), and P (Δ8–254). They were expressed transiently in CHO cells and purified by affinity chromatography. Using soluble fibrinogen degradation products as a t-PA stimulator, they confirmed the work of van Zonneveld et al and showed that (*a*) all the variants containing the kringle 2 domain had nearly normal stimulation and (*b*) all the variants displayed a similar response to increasing concentrations of the stimulator, whereas the protease alone exhibited virtually no stimulation. They also analyzed t-PA binding to forming fibrin clots. Variants that lacked the finger domain but contained the kringle 2 domain were less avid than the wild type. If plasminogen was present, however, the variants lacking the finger domain bound significantly better than in the absence of plasminogen, and this binding approached normal levels when clots were made with physiological concentrations of fibrinogen. The independent protease domain appeared not to interact with fibrin. These

results suggest that although both the finger and the kringle 2 domains have fibrin-binding sites, only kringle 2 is required for optimal fibrin stimulation. This may be because plasminogen is able to increase the binding of variants containing kringle 2, by forming a ternary complex of the activator, plasminogen, and fibrin as described previously (1), or because of the ability of plasmin to degrade the fibrin matrix slightly to create new binding sites for both plasminogen and t-PA (76–80).

Bang et al (81) constructed a variant that lacked both kringles (FGP Δ87–261). This variant had a similar specific activity to that of t-PA (on a weight basis) in plasminogen activation assays in both the presence and absence of fibrin (81). However, it was inhibited less effectively than t-PA was by PAI-1. In addition, the biological properties of the variant were tested in a Chandler loop system (82). A whole-blood clot was suspended in a chamber and perfused with circulating plasma. When equivalent activities of t-PA and the variant (but more variant on a molar basis) were infused, the variant appeared to cause significantly more thrombolysis with equivalent amounts of fibrinogen and plasminogen breakdown, possibly because its smaller size allowed for more efficient diffusion into the clot.

Other groups made the converse K1K2P (Δ6–86) variant as well as a specific finger deletion GK1K2P (Δ6–50) and growth factor deletion FK1K2P (Δ51–86). These deletions were made at specific intron/exon boundaries and were tested both in vitro (83, 84) and in vivo (85). The specific plasminogen-activating activity in the presence of fibrin fragments was found to be 525,000, 405,000, 355,000, and 320,000 IU mg of protein^{-1} for the wild type, GK1K2P, FK1K2P, and K1K2P, respectively (83). These values were based on compositional amino acid analysis, which proved to be much more accurate than ELISA in determining the concentration of the proteins lacking the finger domain. None of the variants bound well to fibrin, and the addition of plasminogen had no effect on the binding, in contrast to a similar GK1K2P variant described by Verheijen et al (75). The fibrinolytic potential of the three variants was measured by using a radioactive fibrin clot lysis assay in plasma. At low concentrations (15–50 IU ml^{-1}), all of the variants exhibited significantly lower activity than wild-type t-PA did. At higher concentrations (300–500 IU ml^{-1}), the variants exhibited a level of activity that was identical to or slightly higher than that of wild-type t-PA. The decreased activity at low concentrations was also observed in a purified system; this suggests that protease inhibitors are not involved in this phenomenon. The in vivo results with these variants and both in vitro and in vivo results with multiple variants that also lack carbohydrate attachments sites are discussed below.

Kalyan et al (86) also made a K1K2P (Δ1–89) variant and analyzed it in similar assays following expression in mouse fibroblast C-127 cells. They found, as had Larsen et al (83), that the variant did not bind well to fibrin.

However, they found a much more dramatic effect (a decrease of about 85% compared to wild-type t-PA) on the ability of low concentrations of the variant to activate plasminogen in the presence of soluble fibrin. At higher concentrations of activators the activity of the variant was comparable to the activity of wild-type t-PA. The activity of K1K2P was near normal in the absence of a stimulator and against small synthetic substrates. In their plasma clot lysis assay (86) the K1K2P variant was virtually ineffective, in agreement with results of other workers (83). However, even at higher levels, about sevenfold more K1K2P than wild-type t-PA was required for complete lysis. The reason for this discrepancy is unclear at present.

Another group (87) made a complete series of domain deletions and obtained different results regarding the influence of the kringle 1 domain than had been obtained by previous workers. This series contains GK1K2P (Δ4–50), FK1K2P (Δ50–88), K1K2P (Δ4–88), FGK2P (Δ92–179), FGK1P (Δ177–261), FGP (Δ87–262), and P (Δ4–262). The deletions correspond to the intron/exon junctions. All of the variants exhibited similar activity against small-peptide substrates and in plasminogen activation assays in the absence of a stimulator. In plasminogen activation assays in the presence of soluble fibrin (Desafib®) as a cofactor, FK1K2P, FGK1P, and FGK2P were indistinguishable from wild-type t-PA. K1K2P failed to exhibit Michaelis-Menten kinetics over the concentration range of lys-plasminogen used in the assays, and GK1K2P had a slightly higher K_m for plasminogen. However, these variants with either kringle 1 or kringle 2 or both kringles exhibited stimulation equivalent to that of wild type t-PA, unlike the FGP and P variants, which were very poor plasminogen activators in the presence of stimulator. These results suggest that either kringle is sufficient for optimal stimulation. Morcovcr, only whcn both kringles were gone (i.e. in the FGP or P variants) were the proteins incapable of binding to lysine-Sepharose. All other variants bound efficiently to the column. They also found that all of the variants were inhibited by PAI-1, which suggests that the protease alone is necessary for interaction with the inhibitor.

Rehberg et al (88) constructed a plasminogen activator gene with unique restriction sites to facilitate the manipulation of various domains. This "designer" t-PA gene with unique restriction sites codes for a protein with several altered amino acids. The t-PA analog contains an Arg inserted between residues 1 and 2, and a Ser-Val inserted between residues 176 and 177, as well as the substitutions K49N, S50A, E85D, S262A, and L266R and the deletions ΔS178 and ΔQ268. This t-PA analog was found not to be significantly different from either wild-type recombinant t-PA or Bowes melanoma cell t-PA in its ability to activate plasminogen in the presence of a stimulator, although it was slightly more active in the absence of a stimulator. It was used as the background for the domain deletions GK1K2P, FK1K2P, FGK1P, and

FP, as well as the kringle swap mutant FGK2K1P. All the variants were capable of hydrolyzing a small synthetic substrate. Kinetic analyses of the GK1K2P and FGK1P variants in the absence of a stimulator indicated that the only difference from wild type was a 2-fold increase in the K_m with GK1K2P (89). In the presence of fibrinogen fragments, GK1K2P had a k_{cat} that was decreased about 10-fold and a K_m that was increased about 4-fold (89). With FGK1P the k_{cat} was decreased about 50-fold without any significant effect on the K_m; this led the authors to conclude that the finger domain contributes to the affinity of t-PA for glu-plasminogen and that the kringle 2 domain influences the catalytic rate constant in the presence of a stimulator. The variants were all inhibited similarly by PAI-1 when the variant concentration was standardized by plasminogen-activating activity in the presence of a cofactor (90). The same group investigated the ability of the some of the variants to lyse human whole-blood clots and bind to fibrin (91). They found that the order of activity was t-PA >> FK2P > FK1K2P > GK1K2P > FP, which was parallel to the order of fibrin binding.

IN VIVO RESULTS The most dramatic biological difference that has been observed for domain deletion variants is their prolonged half-life in vivo. The pharmacokinetics of two similar, but not identical, K1K2P variants in rabbits (85) as well as in mice (86, 92) has been tested. K1K2P (Δ6–86) had a half-life of about 15 min in rabbits, compared with 5 min for wild-type t-PA (85). The alteration of the K1K2P (Δ1–89) variant in mice appeared more pronounced; wild-type t-PA had a half-life of 2 min; however, the half-life of the variant was about 50 min (86). When the plasma disappearance profile of this variant was analyzed more closely (which points out some of the problems in the analysis and comparison of pharmacokinetic data), it was evident that the elimination of K1K2P was biphasic (92). The use of clearance rates is the most informative way of quantifying the elimination. These calculations yield a clearance of 4.2 ml min^{-1} kg^{-1} for t-PA and 0.48 ml min^{-1} kg^{-1} for K1K2P (92). It is difficult to discern whether the differences reported between the two K1K2P variants Δ1–89 and Δ6–86 reflect true differences in the clearance that are dependent on the exact amino acids present in the variant or whether they might be due to differences in the expression systems used, the species for which the half-life was measured, or the analysis of data. It should also be noted that the authors found that the majority of the variant protein remaining in plasma was in the form of a high-molecular-weight complex (92). The complex was not detected with wild-type t-PA, which was cleared from the plasma more rapidly. It is tempting to conclude that the difference in the clearance is due to the deletion of a portion of the protein responsible for the binding to a liver receptor, although effects on the conformation of the protein cannot be excluded.

Variants with only the finger domain deleted, GK1K2P (93, 94), or with only the growth factor domain deleted, FK1K2P (94, 95) have also been shown to have decreased clearance rates in rabbits, rats, and guinea pigs. The GK1K2P (Δ1–44) variant had a clearance that was 20-fold lower than that of wild-type t-PA in rabbits (93). In rats, GK1K2P (Δ6–50) disappeared in a single phase (unlike the wild type, which exhibits biphasic disappearance) with a half-life that was also increased ca. 20-fold (94). When acylated at the active site, the FK1K2P (Δ51–87) variant was also cleared much more slowly (95) in guinea pigs; this result was obtained by measuring the residual activity in plasma. Unfortunately, clearance parameters were not reported, but the half-life of the initial phase appears to be about 4-fold longer. The half-life of the secondary phase was also prolonged significantly. When the pharmacokinetics of a very similar FK1K2P variant (Δ51–86) in rats was measured without acylation of the active site, the half-life of the first phase was prolonged about 2.5-fold (94). Although the half-life of the secondary phase was slightly shorter with the variant than with the wild type, the area under the curve was increased about 4-fold overall with FK1K2P. These studies indicate that the finger domain is a much more important determinant of clearance than the growth factor domain.

The K1K2P (Δ6–86) variant has also been compared with melanoma t-PA in the rabbit jugular vein thrombolysis model (85). The two proteins gave similar thrombolysis over the dose ranges tested (0.13–0.50 mg kg^{-1}), although the concentration of antigen in plasma with the variant was 30- to 40-fold higher than with wild-type t-PA. This suggests that the specific thrombolytic activity of the K1K2P variant, when based upon the antigen concentration achieved, is actually lower than with wild-type t-PA. In addition, significantly more fibrinogen and α_2-antiplasmin were consumed with the variant, suggesting that it is less fibrin specific. These results fit well with the in vitro data, which indicate that the variant does not bind well to fibrin and is not stimulated as effectively by fibrin as the wild-type.

Carbohydrate Variants

Before the use of molecular biology approaches to study the influence of carbohydrate on the function of t-PA, investigators used either protein expressed in the presence of a glycosylation inhibitor such as tunicamycin or protein from which the carbohydrate side chains were enzymatically removed (96). It was observed that the removal of the three carbohydrate side chains had minimal effect on the function of the molecule. Similar approaches combined with site-specific mutagenesis have been used to examine the effect of carbohydrate on the in vivo clearance of the molecule. Hotchkiss et al (97) found that either periodate oxidation or the more specific endo-β-*N*-acetylglucosaminidase H (endo H) treatment of t-PA resulted in a decrease in

clearance of approximately threefold. Since endo H removes only the high-mannose-type carbohydrates present at position 117 on t-PA, the authors also constructed a variant (N117Q) without a carbohydrate at that position. The decreased clearance of this variant, combined with the observation that t-PA was more rapidly cleared if expressed in a cell line which produces only high-mannose oligosaccharide structures, confirmed that the clearance of t-PA is determined in part by a mannose-dependent mechanism. These changes (maximum threefold) are clearly not as dramatic as the changes observed with the deletion variants described above. Lau et al (98, 99) also removed the complex carbohydrate structure at position 117 on the protease domain via site specific mutagenesis. (Note that the numbering convention used in this review differs from that used by Lau et al.) The N117Q variant exhibited slightly higher specific activity in amidolytic and plasminogen activation assays. A more dramatic difference between N117Q and wild-type t-PA was evident in the half-life. In an experiment with rabbits, in which amidolytic activity was used to quantify the t-PA remaining, the wild-type protein showed monophasic disappearance from plasma with a half-life of 3 min, whereas the clearance of the variant was clearly biphasic, with an initial half-life of 0.9 min and a secondary half-life of 27 min. Clot lysis in the in vivo rabbit jugular vein model was also monitored, and it was confirmed that the prolonged half-life of the N117Q variant correlated with increased thrombolysis. The carbohydrate at position 184, which is present in only about 50% of the molecules obtained by using mammalian expression systems, has been implicated in several in vitro functions of the molecule. Type II t-PA without the carbohydrate in position 184 has been shown to exhibit slightly higher fibrin binding (Paoni and Keyt, unpublished observations) and increased specific plasminogen-activating activity in the presence of a fibrin cofactor (24, 100).

Domain Deletion and Carbohydrate Double Variants

Variants combining domain deletions with carbohydrate changes are quite interesting because of the implication of the finger and growth factor domains, as well as the carbohydrate residues, in the biological half-life of t-PA. Two different combination variants have been tested, an N117Q K1K2P (Δ6–86) variant, in which only the site of attachment for the simple sugar was removed, and an N117Q N184Q N448Q K1K2P (Δ6–86) variant, in which all three carbohydrate attachment sites were removed (85). When tested in vitro, both of the K1K2P variants that lacked carbohydrate attachment sites had higher specific activities (~2- to 3-fold) on fibrin plates. In a fibrin-binding assay, N117Q K1K2P exhibited similar binding to that of K1K2P; however, the variant lacking all three carbohydrate attachment sites showed enhanced binding (although it was still decreased when compared with that of wild-type

t-PA. Presumably this is due to the added exposure of the binding site on kringle 2, which is unmasked upon the removal of the carbohydrate at position 184. The clearance of these variants was compared with the clearance of wild-type t-PA and K1K2P with all of the carbohydrate attachment sites intact. They were cleared at different rates, with initial half-lives of ~4, ~14, ~25, and ~42 min for the wild type, N117Q N184Q N448Q K1K2P, K1K2P, and N117Q K1K2P, respectively. The explanation for this rank ordering of variants is not well understood, since the removal of the high-mannose attachment site at position 117 seems to have the most significant effect on the clearance. Notably, however, this effect is decreased significantly when the other two carbohydrates are also removed. When all were tested in the rabbit jugular vein thrombolysis model, the N117Q N184Q N448Q K1K2P variant behaved similarly to K1K2P and wild-type t-PA, indicating that the alteration in the half-life is almost completely counteracted by an effect on the potency with which the variant activates plasminogen at the site of a thrombus. The N117Q K1K2P variant was more effective at lower doses, however, suggesting that the decreased clearance was able to increase the potency, even though the specific activity on a plasma level basis was decreased somewhat. When tested in dogs (101), the clearance of the N117Q N184Q N448Q K1K2P variant was ~20-fold lower than the clearance of wild-type t-PA. In the copper coil–induced coronary artery thrombosis model, the K1K2P variant devoid of all carbohydrate was an effective thrombolytic agent when given as a bolus; melanoma t-PA was ineffective as a bolus, presumably owing to its shorter half-life (101).

Variants Resistant to the Conversion from One Chain to Two Chains

Several groups (102–104) have replaced the arginine at position 275 with other amino acids to investigate the properties of a form of t-PA which remained one-chain even after catalytic quantities of plasmin were produced. The three investigations found somewhat different properties in the variants analyzed. The first of these one-chain variants investigated, R275E t-PA (102), exhibited full plasminogen-activating activity in the presence of a fibrin(ogen) cofactor, although its plasminogen-activating activity in the absence of cofactor was about 20-fold lower than that of two-chain t-PA. The activity of R275E t-PA against small synthetic substrates was also decreased, and this decrease was primarily due to an increase (~10-fold) in the K_m of the reaction with the one-chain form of the enzyme compared with the two-chain form of wild-type or two-chain R275E t-PA that had been converted with V8 protease. Similar results were found with an R275G variant (103) in a plasminogen activation assay in the absence of a fibrin(ogen) cofactor. The authors found that in the presence of the cofactor, the activity increased to within two-fold of the activity found with two-chain t-PA. However, in an

assay against small-peptide substrates, they found that the conversion from the one-chain form to the two-chain form resulted primarily in an increase (~8-fold) in the k_{cat} of the reaction. In addition, they determined that fibrin could enhance the activity of the one-chain wild-type or variant t-PA against small synthetic substrates. This increase was due to an increase in the k_{cat} of the reaction. These results suggest that fibrin is capable of inducing a conformational change similar to that caused by proteolytic hydrolysis to the two-chain form. Boose et al (104) also made the R275G variant and found a 3-fold increase in the k_{cat} of the reaction against a different peptide substrate when the one-chain form was converted to the two-chain form. They also found that the one- and two-chain forms of the molecule differed in their abilities to convert plasminogen to plasmin, with the two-chain form having a k_{cat} approximately 4-fold higher. Fibrin could stimulate the conversion with both two-chain t-PA and the one-chain variant, although ca. 10-fold more fibrin was needed with the one-chain variant and its maximal activity was about half that obtained with two-chain t-PA.

Other position 275 t-PA variants have been reported: R275D (105); R275H, R275K, and R275T (106); and all 19 possible substitutions (107). The R275K variant (106, 107) is plasmin sensitive and behaves similarly to wild-type t-PA. There is disagreement about whether R275H is plasmin sensitive (106) or not (107). All other position 275 variants behaved similarly to R275E as described above, except for R275C, which had significantly lower plasminogen-activating activity. This reduced activity may be due to improper protein disulfides (misfolding) or a mixed disulfide formation resulting from the additional thiol present at Cys275 (107). The R275E variant was also tested for thrombolytic activity in primates and rabbits and found to be active in vivo (108). Clearance was measured by monitoring the plasma levels obtained during infusion and was 2.2- and 1.7-fold slower than the clearance of wild-type t-PA in primates and rabbits, respectively. The rate and extent of thrombolysis in the same species were measured, and dose-response curves were used to determine the ED_{40} (dose for 40% clot lysis). In primates the ED_{40} was 870 $\mu g\ kg^{-1}$ for for wild-type t-PA and 680 $\mu g\ kg^{-1}$ for R275E, whereas in rabbits the ED_{40}'s were 740 and 660 $\mu g/kg$ for wild-type t-PA and R275E, respectively. These data indicate that although R275E is more potent than wild-type t-PA at a given dose, this increase is due to the decreased clearance of the variant. When potency is determined on the basis of plasma concentrations, the potency of the variant is actually decreased somewhat compared with the wild type.

Other Site-Specific Variants

Several other groups have made limited site-specific variants designed to alter specific properties of t-PA. Madison et al. (109) made several variants with alterations from amino acids 296 to 304. Residues 296–302, which corre-

spond to a loop unique to t-PA and u-PA, were deleted. In other variants, t-PA residue R304, which corresponds to a residue in trypsin thought to interact with proteinaceous inhibitors, was changed to serine or glutamic acid. All three variants were found to react less well with PAI-1: under conditions where the activity of wild-type t-PA could be completely inhibited by PAI-1, 95% of the Δ296–302 t-PA activity remained. Mutants R304S and R304E required 4- and 25-fold more PAI-1, respectively, for half-maximal inhibition of activity compared with wild-type t-PA. All variants maintained similar plasminogen levels in the presence of a stimulator, with k_{cat}/K_m values within 2-fold for all of the variants. The present lack of in vivo data for these variants makes it impossible to discern whether resistance to PAI-1 inhibition will influence the thrombolytic activity of t-PA. Another group made site-specific deletions in the carboxy-terminal portion of the molecule and combined these with carbohydrate variants (110). They found that Δ524–527 t-PA had increased activity in a fibrin-dependent plasminogen activation assay, and when combined with N117Q and N184Q, the variant exhibited a 6-fold increase in activity. In addition, this variant had decreased reactivity with PAI-2, as did two other variants (K277I and K277R) made by the same group. Unfortunately, no tests on clot lysis in vivo have been reported for any of these variants at the time of this writing.

Chimeras with Related Proteins

Hybrid molecules between proteolytic domains from plasminogen activators (t-PA or u-PA) and amino-terminal domains from proteins that bind fibrin (t-PA and plasminogen) or from nonbinding proteins (u-PA) have been used both to dissect the function of the various domains and to try to engineer a protein with improved properties. Chemical, enzymatic, and genetic engineering approaches have been used to construct these chimeras. A hybrid containing the amino-terminal domains of plasmin (K78–R561) and the protease domain of u-PA (I159–L411) was produced by limited reduction and affinity chromatography, followed by reoxidation (111). Although the hybrid did bind to fibrin and exhibited a 5-fold enhancement of plasminogen-activating activity in the presence of soluble fibrin, the amidolytic activity of the hybrid was ca. one-half that of high-molecular-weight u-PA in terms of international units per milligram. A similar approach was used to prepare a variant with the t-PA protease attached to the plasminogen amino-terminal fragment (112). This hybrid also showed decreased amidolytic activity (~40% of normal). It bound to fibrin, but to a lesser degree than melanoma t-PA did. The plasminogen-activating activity was difficult to quantify owing to nonparallel dose-response curves, but appeared to be slightly lower than for melanoma t-PA. More recently, the same group has analyzed plasminogen activation kinetics of the plasminogen–u-PA and plasminogen–t-PA hybrids

(113). The k_{cat}/K_m values of the chimera in the absence of a stimulator were about 6- and 16-fold lower than similar constants for the u-PA or t-PA parent molecules, respectively. The presence of cyanogen bromide fragments of fibrinogen had little effect on the plasminogen activation by u-PA or its chimera, but increased the k_{cat}/K_m value for t-PA and its hybrid 7- and 12-fold, respectively.

Chimeric proteins have also been made by recombinant DNA techniques. Similarities between the domains of t-PA and u-PA have led several groups to combine the domains of the two plasminogen activators by fusing the appropriate coding sequences of the cDNAs. A chimeric protein containing the amino-terminal half of t-PA (S1–T263) attached to the protease domain of u-PA (L144–L411) was expressed in the one-chain form in mammalian cells and characterized (114). Similarly to one-chain u-PA, this hybrid had significantly decreased amidolytic activity in the one-chain form, but when it was converted to the two-chain form, its activity increased more than 100-fold. Both forms of the hybrid bound to fibrin (although not as efficiently as t-PA did). The plasminogen-activating activity of the two-chain form of the hybrid was enhanced ~20-fold over the one-chain form of the hybrid. The presence of a fibrinogen-derived cofactor increased this about 100-fold more in the two-chain form. This variant contains the u-PA "activation site" and resembles u-PA in its one-chain, two-chain behavior. Another hybrid, which was fused at the normal position of activation from one-chain to two-chain t-PA, was produced (115). This t-PA/u-PA chimera containing amino acids 1–274 of t-PA fused to amino acids 138–411 of one-chain u-PA had a longer linker region connecting the domains from the two proteins. The chimera also contained the single point mutation C264G on the t-PA segment to remove the new unpaired cysteine residue present in the chimera. The hybrid in either the one- or the two-chain form bound to fibrin, but to a lesser extent than t-PA did. In a [^{125}I]fibrin plasma clot lysis assay, the hybrid had activity similar to u-PA and significantly lower than that of t-PA. In the one-chain form, however, less fibrinogen was degraded than in the two-chain form, as was the case with u-PA. Thus, the conversion of this hybrid from the one- to the two-chain form yields behavior patterns similar to those obtained when one-chain u-PA is converted to two-chain u-PA. This is also observed in inhibition in human plasma or with synthetic inhibitors in purified systems (116).

Two similar variants, 1–262 of t-PA attached to 147–411 of u-PA and 1–262 of t-PA attached to 134–411 of u-PA, were also measured with respect to fibrin binding, fibrin stimulation, and inhibition by PAI-1 (117). As noted by others (114, 115), the fibrin binding of the hybrids was significantly decreased compared with that of rt-PA, although some binding was evident, unlike with u-PA, which does not interact with fibrin. The catalytic efficiency

was increased 2000-fold for rt-PA, ca. 250-fold for the hybrids, and 12-fold for u-PA in the presence of cyanogen bromide fragments of fibrinogen. Surprisingly, the K_m's in both the absence and presence of the fibrinogen cofactor were lower with the hybrids than with t-PA and u-PA, which yielded an increased k_{cat}/K_m. The discrepancy between this result and others in the field is difficult to rationalize, since it is difficult to explain why a hybrid of this sort would have an improved reactivity with the substrate when compared with either of the parent molecules. It is quite feasible that there might be difficulty in quantifying the chimera. However, a monoclonal-monoclonal ELISA in which both monoclonal antibodies are directed against the kringle 1 domain of the molecule was used. Possibly the most interesting results were obtained when the reaction with PAI-1 was measured. Under conditions where the relative affinity of PAI-1 for rt-PA was ~4.6-fold greater than the affinity for u-PA, the affinity for the 1–262 t-PA attached to 124–411 u-PA was only slightly less than that for rt-PA, whereas the affinity for the 1–262 t-PA attached to 134–411 u-PA hybrid was less than that for u-PA itself. This indicates that barring an effect on the conformation of the protease domain, the linker region between the protease and kringle structures has an effect of the interaction with the inhibitor.

Another similar variant, 1–261 from t-PA attached to 132–411 from u-PA, exhibited properties similar to both u-PA and t-PA (118). Like u-PA, the chimera exhibited a high intrinsic plasminogen-activating activity in the absence of fibrin. Like t-PA, its activity was stimulated by fibrin, but only 3-fold, compared with 60-fold for intact t-PA. This stimulation was determined to be primarily from a drop in the K_m of the reaction. The chimera was also capable of binding to a fibrin clot, as is t-PA but not u-PA.

Other similar variants, 1–262 from t-PA attached to 139–411 from u-PA, 1–67 of t-PA attached to 136–411 of u-PA, and 1–313 of t-PA attached to 195–411 of u-PA, were also made (119). Unfortunately, there were three single-amino-acid changes in the u-PA clone, one of which removed a cysteine residue normally present in a disulfide bond and one of which created a new, presumably unpaired cysteine residue. These alterations were corrected in some of the expression vectors. All of the variants had lower specific plasminogen-activating activity than u-PA did. The variant combining 1–67 of t-PA and 136–411 of u-PA was characterized further (120). Unfortunately, this variant, containing the finger and half of the growth factor region of t-PA and the protease portion of u-PA, also contained two point mutations in the u-PA segment: G366C and A410V. The effect of the introduction of a new unpaired cysteine residue (u-PA position 366) in the protease portion of the molecule, in addition to the new unpaired cysteine at position 56 in the t-PA growth factor region as a result of the deletion of its normal partner at position 73, cannot be determined. It is quite possible that these cysteine residues

result in the formation of abnormal disulfide bonds and an altered conformation. The hybrid had a specific activity of ~50% that of u-PA on fibrin plates and exhibited somewhat decreased activity in a [^{125}I]fibrin clot lysis assay in plasma. The t-PA finger domain in the hybrid did not appear to confer any fibrin-binding properties on the hybrid, and in all respects it appeared more similar to u-PA than to t-PA.

Chimeric molecules that contained the t-PA kringles and protease with various sections of u-PA were also produced (121). One hybrid (hybrid A) contained the finger and growth factor domains of u-PA (residues 1–131) attached to the kringles and protease of t-PA (92–527); another hybrid (hybrid B) contained the entire t-PA sequence with the kringle of u-PA residues 50–131) inserted before (residue 92) the kringles of t-PA; a third hybrid (hybrid C) contained the entire sequence of t-PA with the kringle of u-PA (residues 50–131) inserted after (residue 261) the kringles of t-PA. The amidolytic activity of all of the hybrids was similar to that obtained with rt-PA, and all could activate plasminogen and were stimulated by fibrin. However, none of the hybrids were stimulated as effectively as rt-PA. The order of fibrin-binding activity was the same as the order of plasminogen-activating activity, i.e. rt-PA > hybrid B > hybrid C > hybrid A. Assuming that no alterations in the conformation of the molecule caused these effects, it can be inferred that the finger domain is important for both fibrin binding and specific activity in the presence of fibrin.

Other chimeric molecules were produced by inserting an isolated domain(s) from one plasminogen activator into the sequence of the other plasminogen activator. The kringle 2 domain of t-PA (residues 173–262) was inserted between residues 130 and 139 of one-chain u-PA (scu-PA) (122). Residues 131–138 of scu-PA were deleted in this construction. The enzymatic properties of the hybrid in both the one- and two-chain forms were similar to those obtained with one- and two-chain u-PA. The addition of the kringle 2 domain had little effect on either the enzymatic activity or on the fibrin binding of the molecule.

Chimeras with Antibodies

The interaction of t-PA with fibrin is believed to be one of the prime advantages of t-PA as a thrombolytic agent. The binding interaction has been characterized both kinetically (1) and through direct-binding studies (40). The dissociation constant determined both ways is approximately 10^{-7} M, indicating that this is not a high-affinity interaction, although digestion of the clot with plasmin does create new binding sites with affinities which are 2–4 orders of magnitude more tight (40). A novel approach to enhance the affinity is to couple t-PA to a fibrin antibody. Critical to the success of this approach is the ability to produce monoclonal antibodies that specifically bind to fibrin

without showing any reactivity with fibrinogen. Fibrin-specific monoclonal antibodies were produced following immunization with a heptapeptide from the new amino terminus of the β chain of fibrin (123, 124) or the new amino terminus of the α chain of fibrin (124, 125), which are formed following proteolysis by thrombin. Although other groups have described fibrin-specific monoclonal antibodies prepared against the thrombin-digested amino-terminal disulfide knot that contains the amino termini of all of the fibrin chains (126) or the cross-linked fibrin D-dimer (127), the results described in this section were obtained by using antibodies specific to the β chain of fibrin that have dissociation constants ranging from 2×10^{-7} to 7×10^{-8} M (124). These constants were determined by the binding of [^{125}I]antifibrin antibodies to human blood clots. However, it should be noted that the binding affinity was significantly different for at least one of the antibodies when it was measured with a fibrinlike fragment (128).

When u-PA was conjugated to the fibrin monoclonal antibody with a chemical cross-linking reagent (129), enhanced release of labeled peptides from fibrin-Sepharose in the presence of plasminogen was demonstrated. The u-PA–antibody conjugate was effective at about 1% of the dose of u-PA alone. The presence of physiological levels of fibrinogen had no effect of the lysis, although the presence of the β peptide was inhibitory, demonstrating the specificity of this reagent for fibrin. In further studies, Bode et al. (130) determined that the antifibrin Fab' u-PA conjugate was slightly more potent than was the intact antifibrin antibody u-PA conjugate at releasing radioactive peptides from [^{125}I]fibrin-Sepharose. Both conjugates were as effective as u-PA at 0.4% of the concentration as determined by activity in a synthetic-peptide substrate assay. These studies indicate that a univalent complex formed with the Fab' fragment is sufficient for the maximum fibrin specificity and that bivalent complexes (as with intact antibodies) do not appear to confer any increased binding, as measured by the identical activities of the two complexes. In another study from the same laboratory, which involved the use of a different anti-fibrin antibody (131), Bode et al again measured the ability of the chemically cross-linked anti-fibrin Fab' u-PA conjugate to release peptides from [^{125}I]fibrin-Sepharose. At equal concentrations of u-PA (based on the activity against a small synthetic substrate) the conjugate had a potency 95 times that of u-PA. The conjugate also lysed human thrombi in fresh citrated plasma with a maximal potency that was 4.4 times that of uncoupled u-PA.

The ability of a fibrin monoclonal antibody to target u-PA to fibrin and thus increase its potency is not surprising in light of the inability of u-PA to interact with fibrin. Measuring the ability of similar antibodies to enhance the potency of t-PA is a much more challenging experiment, because the affinities of t-PA to fibrin (40) and the monoclonal antibodies to human blood clots (124) are

very similar. Unfortunatcly, the affinities of t-PA and thc anti-fibrin monoclonal antibodies have never been measured under the same experimental conditions. Chemical cross-linking of t-PA to an antibody to fibrin (59D8) with an avidity constant of 1.45×10^7 (dissociation constant of 6.9×10^{-8}) to human blood clots (124) or a dissociation constant of 0.77×10^{-10} (128) based on the binding to a fibrinlike fragment, N-terminal disulfide knot, was used to compare the fibrinolytic potential of this conjugate with that of a similar u-PA conjugate as well as with those of t-PA and u-PA alone (128). Both antibody conjugates were equally effective in the lysis of [^{125}I]fibrin-Sepharose and were 100 times more potent than u-PA alone and 10 times more potent than t-PA alone. Although the binding of the conjugates to fibrin was not measured directly, both conjugates exhibited a 3- to 5-fold-enhanced lysis of human clots in human plasma over that of the respective unconjugated plasminogen activator. The binding of the u-PA conjugate was approximately equal to that of t-PA alone. At equipotent fibrinolytic concentrations, both conjugates appeared to consume less fibrinogen, α_2-antiplasmin, and plasminogen than did the unconjugated activators. The t-PA anti-fibrin antibody conjugate has also been tested in a rabbit jugular vein model of in vivo thrombolysis (132); it was significantly more potent than was t-PA alone. When administered at equal peptidase activities, the conjugate exhibited an average 2.8-fold enhancement of lysis over all concentrations studies. This enhancement was most dramatic (9.6-fold) at the lowest rates of lysis. The in vivo half-life of the conjugate was also compared with that of t-PA alone and found to be significantly longer. Although the majority of the [^{125}I] t-PA disappears from plasma with a half-life of 6 min, conjugate was cleared biphasically with a short half-life ($\sim$10 min) a longer half-life that exceeded the duration of the experiment (6 hr). Thus, the possibility remains that the enhanced potency was directly related to the decreased clearance of the conjugate. To test whether any antibody conjugate was capable of enhancing the potency of t-PA, t-PA was coupled to an anti-dioxin antibody. This conjugatc did not have incrcased potency; however, its half-life was not measured directly. The ability of the t-PA anti-fibrin conjugate to target the t-PA to the site of the clot was also evident from the decreased amount of systemic fibrinogenolysis and α_2-antiplasmin consumption seen with the conjugate compared with t-PA alone.

Another novel approach used by the same group of investigators was to make an antibody molecule specific for both fibrin and t-PA and measure its effect on fibrinolysis (133). The bispecific antibody conjugate was produced by chemical cross-linking of a noninhibitory antibody specific for t-PA to the 59D8 antibody specific for fibrin. In the assay that measures the release of ^{125}I-labeled peptides from fibrin-Sepharose, the potency of t-PA bound to the antibody was approximately 13-fold greater than that of an equivalent amount

of t-PA alone and was relatively equivalent to the potency of t-PA chemically cross-linked to the same fibrin antibody (128). This increase in potency was similar whether the t-PA was mixed with the antibody conjugate prior to the addition to fibrin or after the antibody conjugate and the fibrin were mixed (133). The ability of the antibody conjugate to "capture" t-PA and localize it to the fibrin at the low concentrations of t-PA (ca. 5 ng ml^{-1}) present in plasma was also tested in a buffer system. Enhancement of fibrinolysis at all concentrations was observed in the presence of a bispecific antibody, with the greatest enhancement (approximately 10-fold) being observed at the lowest t-PA concentration (0.3 ng ml^{-1}). In the plasma clot lysis assay, the bispecific antibody complex with t-PA also enhanced the rate of lysis over that for t-PA alone. Paradoxically, in this assay the greatest effect was observed at the highest t-PA concentration and no enhancement was observed below about 50 U (~100 ng ml^{-1}). Analysis of the plasma samples indicated less consumption of fibrinogen and α_2-antiplasmin with the bifunctional antibody, suggesting enhanced fibrin specificity. These antibodies were tested in the in vivo rabbit jugular vein thrombolysis model (133). Infusion of 10,000 U of t-PA alone resulted in no significant lysis over that in a control. However, if this level of t-PA was infused simultaneously with 2 mg of the bifunctional antibody, lysis was increased from 14% ± 8% in the control to 42% ± 14%. Neither group of animals suffered a loss in fibrinogen or α_2-antiplasmin.

A recombinant antibody-targeted plasminogen activator was also produced (134). The heavy chain from the monoclonal anti-fibrin antibody 59D8 was cloned and combined with the protease domain of t-PA. The plasmid encoding this hybrid containing the heavy chain through the C_H2 region attached to the t-PA protease was transfected into hybridoma cell lines that had lost their heavy chains but retained the light chains. This approach allowed the purification of protein which, from its size on sodium dodecyl sulfate-polyacylamide gel electrophoresis, contained the desired hybrid heavy chains properly disulfide bonded to light chains and heavy chains to give a $F(ab')_2$-like molecule. Although the hybrid bound to fibrin less effectively than the original antibody did, the difference was less than 10-fold. The t-PA protease portion was determined to retain 70% of normal activity on a molar basis against a small synthetic substrate. When assayed for its ability to hydrolyze plasminogen at equal amidolytic concentrations to t-PA, it had equivalent activity in the absence of a fibrin stimulator. The chimeric molecule was, however, ineffective in lysing plasma clots (135, 136). This failure has been attributed to the absence of the arginine at position 275, the normal site of proteolytic conversion of t-PA from the one- to the two-chain form. However, given the ability of other one-chain forms of the molecule to exhibit full plasminogen-activating activity in the presence of fibrin (1, 102), this explanation appears unlikely. A second construction containing the heavy and light chains of the

antibody 59D8 and the low-molecular-weight form of u-PA with the plasmin-sensitive bond intact was made by using similar techniques (137). This antibody chimera binds fibrin as well as the parent antibody (59D8) does and is 10- to 50-fold more active in the fibrin monomer–Sepharose assay. The reasons for the relative effectiveness of the scu-PA–antibody chimera and for the relative ineffectiveness of the t-PA–antibody chimera are unknown at present.

CONCLUSIONS

It is approximately 5 years since the first t-PA variants were constructed by using site-directed mutagenesis. Variants that manifest decreased clearance rates in vivo have been identified, however, in each case the specific activity of the variant (on a plasma level basis) is reduced in in vivo clot lysis models. The ability of the longer-lived t-PAs to have a positive effect on clot lysis in humans has yet to be demonstrated. Although more than 50 variants have been analyzed, none has been shown to be superior to wild-type t-PA in animal models. The variants have, however, provided us with additional information on protein-protein interactions in this complex system. Although some may argue that the variants have raised far more questions than they have answered regarding the functioning of individual domains or residues, it is evident that as more data become available, t-PA variants will amplify our understanding of the structure-function relationships among t-PA and its substrates, effectors, and inhibitors.

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