Design of Novel Thrombolytic Agents Via Domain Modifications*1

LAURENCE A. ERICKSON AND KEITH R. MAROTTI

Cardiovascular Diseases and Molecular Biology Research, Upjohn Laboratories, Kalamazoo, Michigan

ABSTRACT

Generation of plasmin in the vicinity of a blood clot has proven to be an effective approach for treating thrombotic disorders, particularly myocardial infarction. Conceptually, the ideal thrombolytic agent would initiate the formation of plasmin, primarily in association with fibrin incorporated into the occlusive thrombus. Thus, thrombolytic agents that exhibit relative fibrin specificity and, thus, presumably clot selectivity (e.g., tissue plasminogen activator) were expected to have a marked clinical benefit compared to agents that do not display affinity for fibrin (e.g., streptokinase). However, results obtained recently from clinical trials indicate that these 2 agents essentially were equally effective in treating myocardial infarction. With these findings in mind, efforts are being made to develop novel thrombolytic agents that might achieve more rapid and specific thrombolysis than that achieved by presently available agents and, thus, could be administered earlier because of an improved margin of safety. The available data suggest that tissue-type PA (tPA) mutants possessing resistance to endogenous inhibitors, altered fibrin affinity, and/or slower rates of clearance may prove beneficial in this regard. In addition, adjunctive therapies (i.e., anti-platelet and anti-thrombin compounds) have been found to decrease the time necessary to achieve reperfusion and have reduced rates of reocclusion. These efforts are expected to yield therapeutic agents in the 1990s and beyond that, when administered in combination, would exhibit increased efficacy in the treatment of myocardial infarction and other thrombotic disorders.

Keywords. Atherogenesis; platelets; coagulation; fibrin; thrombosis; myocardial infarction; plasminogen activators; analogs; thrombolysis

INTRODUCTION

Formation of a fibrin-platelet thrombus at the site of an atherosclerotic lesion is now recognized as a key event that precipitates vessel occlusion and the subsequent ischemia associated with an acute evolving myocardial infarction (11). Thus, administration of plasminogen activators (PAs), resulting in the generation of plasmin, the primary fibrin-degrading enzyme in the blood, in the vicinity of the occluding thrombus, has been employed to treat this type of thrombotic disorder (39, 40). Indeed, this approach has been validated in clinical trials in which significantly decreased mortality rates have been documented in patients treated with a thrombolytic agent (24). Presumably, this approach also would prove beneficial in treating thrombosis generally (e.g., venous thrombosis and pulmonary embolism). However, susceptibility of such agents to neutralization in vivo, lack of specificity for the occlusive thrombus and the associated bleeding risk, and short circulating half-lives of most thrombolytic agents currently being used, and the attendant high incidence of reocclusion are still considered impediments to optimal thrombolysis. At least one of these deficiencies is associated with the use of each of the currently available thrombolytic agents, suggesting value in searching for more effective treatments of thrombotic disorders. Before describing the successes and challenges associated with this search, however, a brief outline of the underlying basis for the use of PAs in thrombolytic therapy will be presented.

PHYSIOLOGIC FIBRINOLYSIS AND THROMBOGENESIS

Under normal physiologic conditions, after a blood clot has stemmed the flow of blood subsequent to vessel wall injury and the tissue remodeling
phase of wound healing has commenced, PAs produced primarily by the vascular endothelium initiate the degradation of the fibrin network that solidifies the structure of the thrombus (14, 15). Both tissue-type PA (tPA) and urokinase-like PA (uPA) have been implicated in the processes associated with such repair of vascular injuries. These enzymes are extremely specific for plasminogen, their actions culminating in the dissolution of fibrin via the generation of plasmin (27). However, chronic perturbation of the vessel wall and/or an underlying defect in blood-borne components of the hemostatic system may overwhelm this normal process, leading to the development of a pathophysiologic situation including injury to the endothelium and subsequent generation of a vascular thrombus. This process (i.e., thrombogenesis) may be triggered and perpetuated by several mechanisms, including monocyte/macrophage foam cell development, platelet adhesion and activation, and expression of procoagulant factors. In instances in which such thrombi become occlusive and resistant to physiologic dissolution via the endogenous fibrinolytic system, the use of exogenous initiators of plasmin generation may be used to resolve the occlusions. This approach constitutes thrombolytic therapy.

THROMBOLYTIC THERAPY

Several comprehensive reviews of the various approaches that have been used to treat thrombotic disorders via thrombolysis have been published recently (6, 12). Therefore, an historical perspective of thrombolytic therapy will not be presented. Rather, an overview of the benefits and limitations of streptokinase and tPA will be presented to set the foundation for discussing the generation of novel tPA analogs.

Streptokinase is not a fibrin-sparing agent but has proven to be quite efficacious in clinical trials (24). Whether these results are attributable to other effects of this drug or to the fact that selectivity for the thrombus is not a critical factor in achieving reperfusion of coronary arteries cannot be concluded from published information. In contrast, it is clear that tPA possesses an affinity for fibrin that is not apparent with streptokinase (38). Although the exact mechanism for the stimulation of tPA-mediated plasminogen activation by fibrin and its derivatives is not yet completely understood, it is accomplished through a complex set of interactions involving not only tPA and plasminogen but also fibrin itself (20). Based upon this fibrin-dependent plasmin generation, many have thought that the ideal thrombolytic agent would consist of an absolutely thrombus-specific PA (50). Thus, the concept of "clot selectivity" has emerged, and has been applied to clinical thrombolysis (51). Underlying this concept is the idea that a fibrin-specific agent would induce more rapid thrombolysis than a non-fibrin-specific agent and that there would be less associated bleeding. Thus, tPA's affinity for fibrin and its increased rate of activating plasminogen in the presence of fibrin suggested potential advantages over streptokinase.

In spite of tPA's fibrin selectivity, however, achievement of effective thrombolysis has required the use of doses that also have an associated bleeding risk (50). This result emphasizes potential differences between fibrin stimulation of the activity of tPA under normal physiologic conditions, in which tPA is present at very low concentrations and, in pathologic situations, in which large doses of enzyme are administered over a relatively short period of time. That is, under conditions of thrombolytic therapy, in which circulating levels of tPA may reach concentrations as high as 1 μg/ml, the issue of fibrin selectivity may be less critical. This may be associated with the fact that the expected clinical benefit of tPA has not been fully realized (52).

NOVEL THROMBOLYTIC AGENTS

Efforts to design novel tPA analogs have been undertaken with the hope of improving the therapeutic profile of tPA by increasing or at least maintaining its fibrin specificity, while at the same time eliminating properties that are thought to be undesirable, such as susceptibility to endogenous inhibitors and rapid clearance. Selected examples of such analogs constructed via domain modifications will be presented to illustrate key concepts underlying their design, characterization, and evaluation.

Design

The relationships between the structure of tPA and its function are based partially upon homologies that exist between this enzyme and other factors involved in blood coagulation and cell-cell interactions (33). These relationships have been the subject of intense study since elucidation of the structure of the tPA gene (8) and the demonstration that the exons (or group of exons) coincide with proposed protein structural domains (30). These findings suggested that tPA is a mosaic that arose from exon shuffling (19). Thus, tPA consists of 5 putative regions: the fibronectin finger domain (F), the epidermal growth factor domain (G), 2 "kringle" domains (K₁ and K₂), and the serine protease domain (P) (1). Conceptually, incorporation of each of these domains into the protein resulted in the acquisition of a new functional property.

To attempt to determine which of the domains of tPA are responsible for its various functions, sev-
eral investigators have produced a variety of mutants via a number of different approaches. For example, the first sets of domain deletion mutants described were generated via utilization of natural restriction sites (45, 46, 49). Subsequently, other investigators have utilized M13 deletion mutagenesis to construct more precise exon deletion mutants (25). A “cassette” gene constructed by chemically synthesizing the DNA encoding tPA and incorporating unique restriction sites within the gene also has been used for generating mutants (3, 36).

Characterization and Evaluation

Utilization of the diverse approaches just outlined emphasizes the importance of fully characterizing the structural attributes of purified tPA derivatives prior to biochemical and biological testing. This relates not only to the integrity of the primary amino acid sequence and glycosylation pattern, but also to determination of the protein concentration of the preparation. These criteria have not been met in all cases in which tPA analogs have been evaluated. With these considerations in mind, we will present selected examples of the properties of certain tPA analogs to attempt to provide a consensus view of the functions of the various domains of tPA.

Catalytic Efficiency and Interaction with PAI-1. The protease or “P” domain of tPA contains the catalytic center and, thus, presumably the primary site of interaction with inhibitors (37). Rehberg et al (36) presupposed that the individual domains of tPA functioned as modular units and, thus, made several amino acid changes in the interdomain regions and also constructed several domain deletions and rearrangements. None of these modifications significantly affected the catalytic activity of the tPA analogs towards a synthetic substrate. This finding supports the “modular” hypothesis as far as the catalytic domain is concerned. Extending this concept considerably, Madison and co-workers (29) identified a region in tPA outside of the active site that appears to interact with the primary endogenous tPA inhibitor, plasminogen activator inhibitor-1 (PAI-1). Specifically, after modeling the active site of tPA to that of trypsin bound to bovine pancreatic trypsin inhibitor, a mutant tPA lacking amino acids 296 through 302 was generated via site-directed mutagenesis. This molecule has all of the in vitro biochemical properties as does native tPA, but exhibits a reduced interaction with PAI-1. Thus, although PAI-1 interacts primarily with the active site of tPA, neutralization of enzymatic activity is dependent upon an additional point of contact between tPA and PAI-1. This finding demonstrates that rather subtle modifications of the structure of tPA may not perturb certain functional attributes of this molecule, but may eliminate or modify others. This principle also is apparent in tPA analogs that have been altered with the hope of identifying the fibrin-binding domain(s) of tPA.

Fibrin Binding and Clot Lysis. As indicated earlier, one of the major features underlying the generation of novel tPA analogs via manipulation of domains has been the concept of fibrin-selectivity. Numerous studies have shown that mutants, having either the F or K2 domains deleted, have reduced binding to fibrinogen (10, 23, 25, 35). Mutants with both of these domains deleted have no apparent affinity for fibrin in vitro. In addition, the K2 domain has been convincingly shown to be the lysine-binding domain of tPA (9, 47), which has implicated this domain in fibrin binding. Binding of tPA to fibrin via K2 is inhibited by ε-amino caproic acid (ε-ACA), whereas binding to fibrin, mediated by the F domain, does not appear to be influenced by this lysine analog. Further, enhancement of tPA’s catalytic activity in the presence of fibrin also has been attributed to the K2 domain (32, 46, 49) in that the activities of mutants lacking K2 were not well enhanced by fibrinogen fragments or fibrin monomers. In addition, deglycosylated tPA and tPA analogs retain their ability to activate plasminogen in the presence of fibrin (18, 28). Heparin binding and stimulation of the activity of tPA by heparin also has been reported. Stein et al (41) have shown that the activity of the mutants FP and FGP is stimulated by heparin but not by fibrinogen fragments, whereas the activity of the K2P mutant is enhanced only modestly by heparin but shows a level of enhancement by fibrinogen fragments comparable to that of tPA. These results demonstrate that the activity of tPA may be modulated by various cofactors via different mechanisms.

In contradiction to the work just described, Getting et al (16) presented evidence suggesting that K1 plays a role in plasminogen activation in the presence of fibrin substitutes. These investigators concluded that either of the kringles endows tPA with an enhanced capacity to convert plasminogen to plasmin in the presence of fibrinogen. However, other investigators have not confirmed this finding (46, 49). Possible explanations for this apparent discrepancy are that somewhat different assays were used in these studies and that different types of samples were employed (i.e., media conditioned by cells vs purified enzymes). Regardless, it seems clear that the kringle structures of tPA enhance the ability of this molecule to activate plasminogen in the presence of fibrinogen.

As an extension of these results, other studies have demonstrated that the fibrin affinity of tPA deletion mutants containing both the F and K2 domains is
not as avid as that exhibited by full-length tPA (17, 25, 47). Moreover, rearrangement of domains has indicated that not only the presence of but also the position of the F and K_1 domains is important for optimal expression of the in vitro activity of tPA (9, 32, 45, 49). These findings indicate that the context in which the domains of the native molecule are arranged also contributes to their functions. Thus, although individual domains appear to possess partially distinct functions, interactions between domains also are critical for the overall function of tPA.

Several tPA deletion mutants have been tested additionally in in vitro clot lysis systems and were shown to possess reduced fibrinolytic potential compared to full-length tPA (25, 44). This reduction in the capacity to induce fibrinolysis paralleled the lower fibrin affinity just described. Nonetheless, it was not clear to what extent these findings were predictive of the efficacy of these analogs in in vivo thrombosis models (e.g., because of altered clearance profiles).

Clearance and Thrombolysis. There is some evidence (although not conclusive) that the G and K_1 domains may be involved in the in vivo clearance of tPA. Browne et al (5) originally found that deletion of the G domain increased the in vivo half-life of tPA by 6- to 10-fold, concluding that the G domain was responsible for clearance. However, a number of investigators have found that other structural changes in tPA also affect in vivo half-life. For example, Hansen et al (18) found that deletion of both the F and G domains increases in vivo half-life, and Lau et al (26) and Hotchkiss et al (21) found that deletion of a single glycosylation site, either on K_1 or on the P domain, decreased clearance rates. Thus, the rapid hepatic clearance of tPA has been attributed primarily to N-terminal domains, in particular the G domain, and, additionally, to the high mannose carbohydrate positioned on K_1. In contrast, Ehrlich et al (13) found that in vivo half-life was increased by deleting both kringle domains but retaining the F and G domains. Taken together, these data suggest that the clearance of tPA is not solely attributable to a particular domain or glycosylation site, but rather is dependent on the overall conformation of the full-length molecule. This lengthening of circulating half-life has led to more efficient in vivo thrombolysis by such tPA analogs, in spite of their reduced fibrin affinity (7).

Collectively, these results indicate that structural domains of tPA encoded by separate exons or sets of exons do not have absolutely separate and distinct functions, but rather exhibit interactive and co-dependent properties (2, 34, 43). As such, they have provided the foundation for initiating in-depth studies of tPA to elucidate more precisely the relationships between the structure, function, and interaction of domains. It is hoped that such efforts will lead to the development of novel tPA analogs containing multiple site-directed mutations (rather than domain deletions and modifications), which may be more efficacious than their natural counterparts.

Future Prospects

The clinical benefits that may be gained by using any one of the numerous novel thrombolytic agents that have been constructed via domain modifications have not yet been realized. At this point, the primary issue that must be addressed is whether or not alterations in susceptibility to inhibition, fibrin-binding (or clot-specificity), and clearance constitute not only a novel, potential advantage but also a measurable medical advantage over currently used agents. The available data indicate that improved clot-selectivity does not translate into decreased bleeding risk. Thus, it is conceivable that a longer acting tPA with modified fibrin affinity may induce more rapid thrombolysis and reduce the frequency of reocclusion as a direct consequence of its prolonged presence in the circulation. At present, the impact that the use of an inhibitor-resistant tPA analog may have on thrombolytic therapy is not yet clear.

The frequency and rate of reperfusion (4), the extent of restenosis and reocclusion, potential bleeding complications, and potential functional improvement depend not only upon the type of thrombolytic agent used for treatment and the timing of administration (42), but also upon the type of lesion present. Thus, obtaining an understanding of the basic biology and biochemistry of "atherothrombosis," coupled with the development of agents that could enhance clot dissolution and maintain vessel patency, are of critical importance to the field of thrombolysis. The therapeutic potential in this area is just now becoming apparent. This is illustrated by the identification of agents that may be useful as "adjunctive" therapies to attempt to accelerate the rate of reperfusion and/or decrease the incidence of reoclusion after thrombolytic therapy. For example, the anti-platelet compound PGE_1 (48) and the thrombin inhibitor argatroban (22) have been shown to enhance thrombolysis or prevent thrombosis in vivo. Selective inhibition of thrombin may be quite a useful approach because thrombolytic therapy has been associated with generation of thrombin activity in the circulation (31). Thus, in addition to the development of novel thrombolytic agents, the application of such adjunctive therapies to the treatment of thrombotic disorders also may provide a significant advance in thrombolytic therapy.
REFERENCES


28. Little SP, Bang NU, Harms CS, Marks CA, and Mat-


