REVIEW ARTICLE

Basic and Clinical Aspects of Fibrinolysis and Thrombolysis

By D. Collen and H.R. Lijnen

BLOOD CONTAINS an enzymatic system called the fibrinolytic system, one of the main functions of which is the dissolution of fibrin clots in the blood vessels. The fibrinolytic system comprises a proenzyme, plasminogen, which can be converted to the active enzyme plasmin by different plasminogen activators (PAs). Two physiologic PAs have been identified, initially based on their immunologic relationship with the PA found in tissues (tissue-type PA [t-PA]) or with the PA found in urine (urokinase-type PA [u-PA]). Inhibition of fibrinolysis occurs at the level of the activators (by PA-inhibitors [PAIs]) or at the level of plasmin (mainly by α2-antiplasmin).

Physiologic fibrinolysis is highly fibrin-specific as a result of specific molecular interactions between PA, plasminogen, fibrin, plasmin, and α2-antiplasmin.

Cardiovascular diseases, mainly comprising coronary artery disease leading to myocardial infarction, cerebrovascular disease causing strokes, and venous thrombosis predisposing to pulmonary embolism and the post-phlebitic syndrome, are a major cause of death and disability. The triggering event in the clinical expression of the acute ischemic event is not the underlying atherosclerotic lesion, but a thrombotic obstruction of the artery. Thus, the common cardiovascular diseases have, as their immediate underlying etiology, thrombosis of critically situated blood vessels with loss of blood flow to vital organs.

One approach to the treatment of thrombosis consists of the pharmacologic dissolution of the blood clot via the intravenous infusion of PAs. Currently, five thrombolytic agents are either approved for clinical use or under clinical investigation in patients with acute myocardial infarction. These agents are streptokinase (SK), two-chain u-PA (t-cu-PA; urokinase), anisoylated plasminogen streptokinase activator complex (APSAC), recombinant t-PA (rt-PA), and recombinant single-chain u-PA (rscu-PA; prourokinase).

Reduction of infarct size, preservation of ventricular function, and reduction in mortality has been obtained with SK, rt-PA, and APSAC. Consequently, thrombolytic therapy has become standard treatment for early acute myocardial infarction. Intravenous SK recanalizes approximately 50% of occluded coronary arteries within 90 minutes and reduces mortality by 25%. rt-PA in combination with adjunctive intravenous heparin is more potent for coronary arterial thrombolysis, producing both more rapid and more frequent recanalization (patency 70% to 75% within 90 minutes). Side effects (mainly bleeding) and the incidence of reocclusion associated with the use of SK and rt-PA (combined with intravenous heparin) are not markedly different. The two comparative megatrials performed to date, the International t-PA/SK mortality trial and the ISIS-3 study, have not shown a difference in mortality between t-PA and SK, but these trials failed to include intravenous heparin administration, which could have blunted the higher efficacy for coronary recanalization of t-PA versus SK by more early rethrombosis in the absence of a lytic state after fibrin-specific thrombolysis. In seven small randomized trials of rt-PA versus nonfibrin-specific thrombolytic agents (see below), in patients with acute myocardial infarction administered immediate intravenous heparin, in-hospital mortality was 43 of 975 (4.4%) in patients administered rt-PA and 71 of 972 (7.3%) in patients administered SK, urokinase, or APSAC (P = .0067).

These findings support the hypothesis, although challenged by the results of the megatrials, that early and sustained coronary artery recanalization is the main determinant of clinical outcome. In the absence of conclusive data on mortality reduction with optimal administration schemes, the choice of thrombolytic agent for the treatment of acute myocardial infarction must therefore, at present, be based on considerations of the lower cost of SK versus the higher efficacy for coronary recanalization of rt-PA.

All available thrombolytic agents still suffer significant shortcomings, including large therapeutic doses, a limited fibrin specificity, and bleeding complications. New developments towards further improved efficacy and fibrin specificity of thrombolytic therapy include mutants of rt-PA and scu-PA, recombinant chimeric t-PA/u-PA molecules, antibody-targeted thrombolytic agents, and combinations of fibrin-dissolving agents with more potent selective anticoagulant or antiplatelet strategies.

Several mutants of rt-PA obtained by deletion/substitution of functional domains or of single amino acids have a markedly reduced clearance, but usually also reduced specific thrombolytic activity (thrombolytic activity per unit steady state plasma concentration), resulting in an unaltered or only marginally improved thrombolytic potency (thrombolytic activity per unit dose administered). Chimeric molecules containing functional domains of both t-PA and scu-PA have intact enzymatic properties of u-PA, partial fibrin affinity of t-PA, and, usually, unaltered or reduced thrombolytic potencies. However, a chimera consisting of amino acids 1 to 3 and 87 to 274 of t-PA and amino acids 138 to 411 of scu-PA, with negligible fibrin affinity, was found to have a 10-fold higher thrombolytic potency than scu-PA in animal models of venous thrombosis, as a result of a delayed in vivo clearance with relatively maintained specific thrombolytic activity.

PAs conjugated with antifibrin or antiplatelet antibodies are targeted to blood clots, resulting in a significantly increased thrombolytic potency.

Properties and mechanism of action of the main components of the fibrinolytic system, clinical aspects of thrombol-
ysis focused on acute myocardial infarction, and new approaches to improve thrombolytic therapy are reviewed below.

**BASIC ASPECTS OF FIBRINOLYSIS**

Fibrinolysis is highly regulated by specific molecular interactions between its components. The physiologic PAs, t-PA and, to a lesser extent, scu-PA, activate plasminogen preferentially at the fibrin surface. Fibrin-bound plasmin is protected from rapid inhibition by α-antiplasmin and may thus efficiently degrade the fibrin of a thrombus. SK, APSAC, and tcu-PA (urokinase), in contrast, activate both circulating and fibrin-bound plasminogen relatively indiscriminately and cause extensive systemic activation of the fibrinolytic system, which may result in degradation of several plasma proteins, including fibrinogen, factor V, and factor VIII. Staphylokinase, a protein obtained from Staphylococcus aureus, was recently found to induce fibrin-specific clot lysis in vitro.9,10

**Plasminogen**

Human plasminogen is a single-chain glycoprotein with a molecular weight (M,) of 92,000 present in plasma at a concentration of 1.5 to 2 μmol/L. It was reported to consist of 790 amino acids with 24 disulfide bridges and it contains five homologous triple-loop structures or “kringles.”11 Subsequently, the cDNA sequence of the plasminogen messenger RNA (mRNA) showed the presence of an extra segment of 790 amino acids with 24 disulfide bridges and it contains five homologous triple-loop structures or “kringles.”11 Subsequently, the cDNA sequence of the plasminogen messenger RNA (mRNA) showed the presence of an extra segment of 790 amino acids with 24 disulfide bridges. The plasminogen molecule contains structures called lysine binding sites that interact specifically with amino acids such as lysine and ε-aminoehexanoic acid. These lysine binding sites mediate the specific binding of plasminogen to fibrin10 and the interaction of plasmin with α-antiplasmin, and play a crucial role in the regulation of fibrinolysis.4,10

**PAs and Their Mechanism of Action**

**t-PA.** Human t-PA is a serine proteinase composed of one polypeptide chain containing 527 amino acids with Ser as the NH₂-terminal amino acid.20 It was subsequently shown that native t-PA contains an NH₂-terminal extension of three amino acids,21 but in general the initial numbering system has been maintained. Limited plasmic hydrolysis of the Arg⁹⁸-Le⁷⁹ peptide bond converts the molecule to a two chain activator held together by one interchain disulfide bond. The t-PA molecule contains four domains: (1) a 47-residue-long (residues 4 to 50) amino-terminal region (F-domain) homologous with the finger domains mediating the fibrin affinity of fibronectin; (2) residues 50 to 87 (E-domain) homologous with human epidermal growth factor; (3) two regions comprising residues 87 to 176 and 176 to 262 (K₁ and K₂ domains) that share a high degree of homology with the five kringles of plasminogen; and (4) a serine proteinase domain (residues 276 to 527) with the active site residues His₂⁹², Asp₄⁷¹, and Ser⁶⁷. The t-PA gene is assembled by “exon shuffling,” as suggested by the observation that the structural domains on the heavy chain (F, E, K₁, K₂) are encoded by a single exon or by two adjacent exons.25 Because of the striking correlation between the intron-exon distribution of the gene and the putative domain structure of the protein, it was suggested that these domains would be autonomous structural and/or functional entities.22,23

T-PA is a poor enzyme in the absence of fibrin, but fibrin strikingly enhances the activation rate of plasminogen. This finding has been explained by an increased affinity of fibrin-bound t-PA for plasminogen (lower Kₘ) without significant alteration of the catalytic rate constant (kcat) of the enzyme.24 Although different kinetic constants have been reported,25 most investigators agree that fibrin stimulates plasminogen activation by t-PA by at least two orders of magnitude. The kinetic data of Hoylaerts et al26 support a mechanism in which t-PA and plasminogen adsorb to a fibrin clot in a sequential and ordered way, yielding a ternary complex. Fibrin essentially increases the local plasminogen concentration by creating an additional interaction between t-PA and its substrate. The high affinity of t-PA for plasminogen in the presence of fibrin, thus, allows efficient activation on the fibrin clot, while no efficient plasminogen activation by t-PA occurs in plasma. Plasmin formed on the fibrin surface has both its lysine-binding sites and active site occupied and is thus only slowly inactivated by α-antiplasmin (half-life of about 10 to 100 seconds); in contrast, free plasmin, when formed, is rapidly inhibited by α₂-antiplasmin (half-life of about 0.1 seconds). The fibrinolytic process thus seems to be triggered by and confined to fibrin.

It was proposed that the initial binding of t-PA to fibrin would be governed by the fibrin domain, and that partial degradation of fibrin would result in enhanced binding of t-PA via kringle 2 to newly exposed COOH-terminal lysine residues.26 Thus, early fibrin digestion by plasmin may accelerate fibrinolysis by increasing the binding of both t-PA26 and plasminogen.27 Optimal binding of t-PA to partially plasmin-digested fibrin was shown to coincide with the generation of fibrin fragment X polymers,28 which are also important for interaction of plasminogen with fibrin.29 Optimal stimulation of t-PA–catalyzed plasminogen activation depends on plasmin-mediated modification of fibrin to desA-fragment X-related moieties, and requires the presence of kringle 1-4 domains in plasminogen.30

**u-PA.** scu-PA (prourokinase) is a single chain glycoprotein with an M₉ of 54,000 containing 411 amino acids.31
Upon limited hydrolysis by plasmin or kallikrein of the Lys198-Ile199 peptide bond, the molecule is converted to a two-chain derivative (tcu-PA, urokinase). The NH2-termin al chain contains a region homologous to human epidermal growth factor (E; residues 9 to 45) and one kringle region (K; residues 45 to 134). The catalytic center is located in the protease domain (residues 159 to 411) and is composed of His304, Asp305, and Ser306. A low M, two-chain urokinase (M, 33,000) can be generated by hydrolysis of the Lys198-Lys199 peptide bond with plasmin. A low M, scu-PA with M, 32,000 (scu-PA-32k) can be obtained by specific hydrolysis of the Glu114-Leu115 peptide bond in scu-PA by an unidentified protease.

scu-PA has a very low reactivity towards low molecular weight synthetic substrates or active site inhibitors that are very reactive towards tcu-PA. In mixtures of purified scu-PA and plasminogen, both tcu-PA and plasmin are quickly generated. The presence and magnitude of intrinsic plasminogen activating potential in scu-PA is still controversial. In the second step, scu-PA (urokinase) has no fibrin specificity and activates fibrin-bound and circulating plasminogen relatively indiscriminately. After the addition of tcu-PA to plasma, extensive plasminogen activation and depletion of α2-antiplasmin may occur, leading to degradation of several plasma proteins. scu-PA, in contrast to tcu-PA, has a significant fibrin specificity, which appears to be mediated by preferential conversion of scu-PA to tcu-PA at the fibrin surface.

SK and APSAC. SK is produced by several strains of hemolytic streptococci; it consists of a single polypeptide chain with M, 47,000 to 50,000 and contains 414 amino acids. SK cannot directly cleave peptide bonds, but it activates plasminogen to plasmin indirectly, after a three-step mechanism. In the first step, SK forms an equimolar complex with plasminogen. This complex undergoes a conformational change resulting in the exposure of an active site in the plasminogen moiety. In the second step, this active site catalyzes the activation of plasminogen to plasmin. In a third step, plasminogen-SK molecules are converted to plasmin-SK complex. The active site residues in the plasmin-SK complex are the same as those in the plasmin molecule. The main differences in the enzymatic properties of plasmin and the plasmin-SK complex are found in their interaction with plasminogen and with α2-antiplasmin. Plasmin, in contrast to its complex with SK, is unable to activate plasminogen, while the plasmin(ogen)-SK complex is virtually not inhibited by α2-antiplasmin.

SK disappears from the circulation with a half-life of approximately 20 minutes. The level of antistreptokinase antibodies, which may result from previous infections with β-hemolytic streptococci, varies largely among individuals. Because SK is inactivated by interaction with antibodies, sufficient SK must be infused to neutralize the antibodies. A few days after SK administration, the anti-SK titer increases rapidly to 50 to 100 times the preinfusion value and remains high for 4 to 6 months, during which period renewed treatment is impracticable.

APSC was constructed with the aim of controlling the enzymatic activity of the plasmin(ogen)-SK complex by a specific reversible chemical protection of its catalytic center (ie, by titration with a p-anisoyl group). APSAC is an equimolar noncovalent complex between human Lys-plasminogen and SK, containing the titrated catalytic center and the lysine-binding sites of plasminogen. Reversible acylation of the catalytic center would thus not affect the weak fibrin-binding capacity of Lys-plasminogen in the complex. Decylation of APSAC, which uncovers the catalytic center, occurs both in the circulation and at the fibrin surface. A plasma half-life of 70 minutes was found for APSAC, as compared with 25 minutes for the plasminogen-SK complex formed in vivo after the administration of SK. Patients with high SK antibody titers (≥100 IU/mL) do not respond to APSAC, and APSAC causes a marked increase in the SK antibody titer within 2 to 3 weeks, which persists for months.

Staphylokinase. Staphylokinase, an M, 15,500 protein produced by S aureus, was shown to have profibrinolytic properties more than four decades ago. The gene coding for the bacterial protein has now been cloned and expressed in Escherichia coli and in Bacillus subtilis. Staphylokinase, like SK, forms a stoichiometric complex with plasminogen and the plasminogen-staphylokinase complex then activates plasminogen following Michaelis-Menten kinetics. In the absence of fibrin, α2-antiplasmin rapidly inhibits the plasminogen-staphylokinase complex, but not the plasminogen-SK complex. Addition of 6-amino-hexanoic acid induces a concentration-dependent reduction of the inhibition rate of the plasminogen-staphylokinase complex. Staphylokinase induces dose-dependent lysis of a 125I-fibrin-labeled human plasma clot submersed in citrated human plasma without causing fibrinogenolysis.

The following mechanism may explain the relatively fibrin-specific clot lysis with staphylokinase in a plasma milieu. In the absence of fibrin, the plasminogen-staphylokinase complex is rapidly neutralized by α2-antiplasmin, thus preventing systemic plasminogen activation. In the presence of fibrin, the plasminogen-staphylokinase complex binds to the clot via the lysine-binding sites of the plasminogen moiety. Thereby its inhibition rate by α2-antiplasmin is markedly reduced, thus allowing preferential plasminogen activation at the fibrin surface. In animal models of venous thrombosis, staphylokinase was shown to be a potent thrombolytic agent with a thrombolytic potency comparable with that of SK. It remains to be established whether staphylokinase may hold promise for fibrin-specific therapeutic thrombolysis.

Inhibitors of the Fibrinolytic System

Inhibition of the fibrinolytic system may occur at the level of plasmin or at the level of the PAs. α2-Antiplasmin is the main physiologic plasmin inhibitor in human plasma, whereas inhibition of the physiologic plasminogen activators t-PA and u-PA occurs mainly by plasminogen activator inhibitor-1 (PAI-1).

α2-Antiplasmin. α2-Antiplasmin is a single-chain glycoprotein with an M, of 70,000 containing about 13% carbohydrate. The molecule consists of 452 amino acids and
contains two disulfide bridges.\textsuperscript{35} α,-Antiplasmin belongs to the serine proteinase inhibitor protein family (serpins). The concentration of the inhibitor in plasma is about 1 μmol/L. α,-Antiplasmin forms a 1:1 stoichiometric complex with plasmin that is devoid of protease or esterase activity. The inhibition kinetics can be represented by two consecutive reactions: a fast, second order reaction producing a reversible inactive complex, which is followed by a slower first-order transition resulting in an irreversible complex. The second-order rate constant of the inhibition of plasmin by α,-antiplasmin (k = 2 to 4 × 10\(^{7}\) M\(^{-1}\) L\(^{-1}\) s\(^{-1}\)) is among the fastest protein-protein reactions described. This high inhibition rate is dependent on the presence of a free lysine-binding site and active site in the plasmin molecule and of the COOH-terminal region and the reactive site Arg\(^{-360}\)-Met\(^{367}\) in the inhibitor. The half-life of plasmin molecules on the fibrin surface, which have both their lysine-binding sites and active center occupied, is estimated to be 2 to 3 orders of magnitude longer than that of free plasmin.\textsuperscript{66}

\textbf{PAI-1.} PAI-1 was first identified in conditioned media of cultured human endothelial cells and rat hepatoma cells, and subsequently in plasma, platelets, placenta, and conditioned media of fibrosarcoma cells and hepatocytes.\textsuperscript{53} It is a single chain glycoprotein with M\(_{r}\) about 52,000 consisting of 379 amino acids. PAI-1 is a member of the serine proteinase inhibitor (serpin) family with reactive site peptide bond Arg\(^{-360}\)-Met\(^{367}\). PAI-1 is the primary inhibitor of t-PA and u-PA in human plasma.\textsuperscript{80} It reacts with single-chain and two-chain t-PA and with tcu-PA, but not with scu-PA or with the SK-plasmin complex.\textsuperscript{68} In healthy individuals, highly variable plasma levels of PAI-1 have been observed, ranging from 6 to 85 ng/mL (geometric mean, 24 ng/mL).\textsuperscript{62} PAI-1 in plasma is stabilized by binding to S-protein or vitronectin.\textsuperscript{63}

\textbf{CLINICAL ASPECTS OF THROMBOLYSIS}

Cardiovascular diseases, comprising acute myocardial infarction, stroke, and venous thromboembolism, are responsible for almost 50\% of deaths in the adult population. These conditions can lead not only to sudden death but also to long-term disability at a large cost to society. The annual incidence of cardiovascular disease in the United States is presently estimated at 1.5 million patients per year with acute myocardial infarction, 1.5 million patients with stroke, and 0.5 million patients with deep vein thrombosis leading to pulmonary embolism.\textsuperscript{64}

The recognition that thrombosis within the infarct related coronary artery plays a major role in the pathogenesis of acute myocardial infarction\textsuperscript{65} or stroke,\textsuperscript{66} and the observation that early administration of thrombolytic agents results in recanalization of occluded coronary arteries,\textsuperscript{67} have provided the basis for the development of thrombolytic therapy in acute myocardial infarction. The hypothesis underlying this form of treatment is that coronary artery occlusion leads to ischemia and cell death, resulting in ventricular dysfunction and reduced life expectancy, and that timely recanalization can prevent cell death, reduce infarct size, preserve myocardial function, and reduce early and late mortality. Clinical trials were therefore designed to: (1) establish patterns of efficacy and safety for thrombolytic agents; and (2) define the real impact of early thrombolytic therapy on mortality. Currently, five thrombolytic agents are either approved for clinical use or under clinical investigation, namely, SK, urokinase, rt-PA, APSAC, and rscu-PA (recombinant pro-urokinase).

\textbf{Mechanism of Clinical Benefit of Coronary Arterial Thrombolysis}

Early coronary artery recanalization, within a time window that allows salvage of ischemic myocardium, most likely is a primary contributor to the preservation of ventricular function and to reduction in mortality. The most rational treatment of patients with acute myocardial infarction is therefore likely to be thrombolytic therapy with agents or combinations that produce persistent recanalization without rethrombosis as frequently and as rapidly as possible with an acceptable level of safety. Several observations suggest that late opening of an occluded coronary artery may also have some beneficial effect. Indeed, late reperfusion may limit left ventricular remodeling, improve the electrical stability of the heart, or provide collateral vessels to viable myocardium.\textsuperscript{68} Furthermore, adjunctive therapy with anticoagulant and/or antplatelet agents may contribute to the efficacy of thrombolysis.\textsuperscript{69}

Whatever the mechanism of action by which thrombolytic therapy affects the survival of patients with acute myocardial infarction, several large scale placebo-controlled trials with intravenous thrombolytic agents have established a significant reduction (of the order of 20\% or more) of both early and late mortality with SK, APSAC, and rt-PA if administered within 4 to 6 hours. The life-threatening side effects, predominantly intracranial hemorrhage and other major bleeding, are not negligible but are consistently less frequent than 1\%. Thus, these studies have been invaluable in showing the beneficial effect of thrombolytic therapy on mortality, although, because of their simple design, they provide little or no information on the mechanisms by which this benefit is achieved.

\textbf{Comparative Properties of Thrombolytic Agents}

Comparative studies between SK and rt-PA have shown a difference in efficacy for early coronary artery recanalization\textsuperscript{70} and this conclusion has been supported by results of several noncomparative studies with similar design and endpoints.\textsuperscript{3} Coronary artery patency is present in approximately 22\% of patients within 90 minutes after the start of placebo infusion (usually heparin and occasionally aspirin), approximately 35\% after 24 to 48 hours, and around 65\% after 1 to 3 weeks. SK administration is associated with about 53\% patency at 90 minutes and 75\% after 1 to 3 weeks, while rt-PA, in combination with intravenous heparin, is associated with 75\% patency at 90 minutes, 85\% at 24 to 48 hours, and 81\% at 1 to 3 weeks.\textsuperscript{4} Although late patency is an important determinant for survival, it is difficult to imagine from these data that in the absence of early patency, it could be a primary parameter of clinical outcome. Early patency, measured approximately 90 min-
utes after the start of therapy is significantly increased over placebo (22%) with both SK (53%) and rt-PA (75%), whereas the efficacy for coronary recanalization with rt-PA is about 50% higher than with SK.

Two megatrials directly comparing SK and rt-PA have not shown a difference in survival.34-37 This lack of correlation between initial efficacy and clinical outcome may have several explanations: (1) that initial recanalization needs to be sustained to convey benefit; (2) that delayed recanalization may be a major contributor to benefit; (3) that the protocols used in the megatrials may have blunted the demonstration of potential differences in clinical benefit; or (4) that there is no direct correlation between early recanalization and clinical benefit, but that other (unknown) mechanisms may be major contributors. Under the first and third explanation, the higher initial efficacy of rt-PA might be offset by more frequent subsequent reocclusion. Several recent trials with angiographic endpoints (discussed below) have indeed shown that, in the absence of conjunctive intravenous heparin, coronary patency after 1 to 5 days in patients treated with rt-PA is significantly lower than that in patients treated with rt-PA and conjunctive heparin.74,76 Indeed, in the absence of heparin, patency rates obtained with rt-PA are similar to those with SK. Consequently, the omission of immediate intravenous heparin in the International t-PA/SK mortality trial8 and the ISIS-3 study75 may have accounted for the comparable clinical outcome. In the second case, recanalization after SK, although initially slower, might catch up. The hypothesis that the initial frequency and the speed of coronary artery recanalization is of only minor importance for the clinical outcome is questioned by the findings in many studies77,78 that mortality reduction is largest in patients treated early. Furthermore, the 65% patency rate after 1 to 3 weeks in placebo-treated patients argues against the predominant role of late patency, in the absence of early patency, for clinical outcome.4 The fourth instance would have to be validated by proper randomized studies.

Role of Heparin in Coronary Thrombolysis With Fibrin-Specific Thrombolytic Agents

The effect of the conjunctive use of heparin in combination with rt-PA on coronary artery recanalization has recently been studied extensively. The results are summarized in Table 1. Whereas heparin did not appear to affect patency at 90 minutes,26 its omission markedly reduced the patency rates measured at 7 to 24 hours,26 48 to 72 hours (mean, 55 hours),26 or at 48 to 120 hours (mean, 81 hours).76 Unfortunately, similar studies with SK are not available, although some effect of heparin might also be inferred from the observed clinical benefit.80 These data indicate that, to obtain the high efficacy rates of rt-PA, concomitant use of intravenous heparin is required, whereas its beneficial effect in association with SK is less well documented.

Impact of Thrombolytic Therapy on Mortality

The results of early and late mortality reduction in placebo-controlled SK trials have recently been summarized.8,8 SK therapy reduces overall mortality within 14 to 30 days by approximately 25%.81 However, in the individual trials, mortality rates in the control groups vary between 6.5% and 13%, and reductions in early mortality vary between 18% and 81%. Clearly, both the large variability in mortality rates in the control groups and the impact of thrombolytic therapy thereon are influenced by patient selection, adjunctive therapy including anticoagulant and antiplatelet drugs, and mechanical coronary interventions.

Two megatrials on thrombolytic therapy with SK and rt-PA have been performed, the International t-PA/SK mortality trial72 and the ISIS-3 trial.73 The aim of the International t-PA/SK mortality trial was to compare the efficacy and safety of 100 mg alteplase (activase; Genentech Inc, South San Francisco, CA) administered over 3 hours, with that of 1.5 MU SK administered over 1 hour, in approximately 20,000 patients with acute myocardial infarction treated with aspirin and beta blockade. In addition, the role of subcutaneous heparin, 12,500 U twice per day, started 12 hours after thrombolytic therapy was evaluated. The primary endpoint, in-hospital mortality, was not significantly different between the SK and rt-PA groups.72 Although the aims and end-points of the trial were simple and straightforward, the subsequent finding that the efficacy of rt-PA for coronary thrombolysis is influenced by the concomitant use of heparin has confused the issue. The ISIS-3 study, reported at the American College of Cardiology meeting in Atlanta, GA, in March 1991, compared the effects of 0.6 MU/kg of duteplase (prolysis; Burroughs-Wellcome, London, UK) administered over 4 hours, 1.5

### Table 1. Influence of Heparin on Coronary Patency After Alteplase Infusion

<table>
<thead>
<tr>
<th>Heparin</th>
<th>Study</th>
<th>Endpoint (time after start of therapy)</th>
<th>Aspirin</th>
</tr>
</thead>
<tbody>
<tr>
<td>With</td>
<td></td>
<td>90 min 7-14 h 48-72 h 48-120 h</td>
<td></td>
</tr>
<tr>
<td>Topol et al, 1989</td>
<td>79 (50/63)</td>
<td>?</td>
<td>No</td>
</tr>
<tr>
<td>Hsia et al, 1990</td>
<td>82 (82/100)</td>
<td>71 (30/42)</td>
<td>Yes</td>
</tr>
<tr>
<td>Bleich et al, 1990</td>
<td>83 (214/258)</td>
<td>?</td>
<td>Yes</td>
</tr>
<tr>
<td>ECSG, 1991</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without</td>
<td></td>
<td>90 min 7-14 h 48-72 h 48-120 h</td>
<td></td>
</tr>
<tr>
<td>Topol et al, 1989</td>
<td>79 (54/68)</td>
<td>62 (48/93)</td>
<td>43 (18/42)</td>
</tr>
<tr>
<td>Hsia et al, 1990</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bleich et al, 1990</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECSG, 1991</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>083</td>
<td>&lt;.001</td>
<td>.015</td>
</tr>
</tbody>
</table>

*The data represent percentage values with absolute numbers given in parentheses.
MU SK over 1 hour, and 30 U of APSAC administered as a bolus. Approximately 45,000 patients with acute myocardial infarction entered the study and all were treated with aspirin. In addition, the contribution of subcutaneous heparin started 4 hours after thrombolytic therapy was evaluated. The results indicated that early mortality was not significantly different with any of the thrombolytic agents, whereas the frequency of “probable” intracerebral hemorrhage was lower with SK (0.3%) than with duteplase (0.7%) or APSAC (0.6%).

Persisting Questions After the ISIS-3 Trial

Irrespective of the problems associated with delayed subcutaneous heparin administration in ISIS-3, there are also problems concerning the dose of the duteplase rt-PA preparation used. Indeed, because both Activase and prolysis are the translation product of the same cDNA (obtained from mRNA of the Bowes melanoma cell line), they could be expected to have identical biologic properties. However, the production and/or purification process appears to have introduced some artifacts in the prolysis preparation, as suggested by the specific activity, which is markedly lower for prolysis (approximately 300,000 IU/mg, based on data provided by Burroughs-Wellcome) than for activase (580,000 IU/mg, based on data provided by Genentech). A dose of prolysis of 0.6 MU/kg body weight thus corresponds to 2 mg/kg body weight. For the average 75 kg patient, this would represent a total dose of 45 MU or 150 mg. Extrapolation of the results obtained with duteplase in ISIS-3 to the standard use of 100 mg or 58 MU of alteplase is not obvious. Indeed, based on the activity of both preparations, the 45 MU dose of duteplase, administered to the 75 kg patient in ISIS-3, would appear to be relatively underdosed vis à vis the 58 MU contained in 100 mg alteplase. Based on the gravimetric amount of material administered in ISIS-3, the average dose of 150 mg of duteplase clearly would be higher than the 100 mg alteplase. In the absence of comparative studies between duteplase and alteplase it remains enigmatic whether their equivalence should be based on biologic activity in the clot lysis assay or on the gravimetric amount. Furthermore, 150 mg alteplase, administered in combination with intravenous heparin, was previously found, in the TIMI-2 trial, to be associated with an intracerebral bleeding rate of 1.6%, which caused cessation of the testing of this dose after several hundred patients, and a reduction of the dose to 100 mg.4,33

In the absence of optimally designed studies with mortality endpoints, comparing SK with rt-PA in the presence of intravenous heparin, it might be of interest to review in-hospital mortality data of smaller comparative trials with rt-PA in combination with heparin, against nonfibrin-specific thrombolytic agents (SK, urokinase, or APSAC). The results of seven trials performed to date are summarized in Table 2. Cumulative in-hospital mortalities were 43 of 975 (4.4%) patients randomly assigned to rt-PA and heparin, and 71 of 972 (7.3%) patients allocated to the nonfibrin-specific agents and heparin. Meta-analysis of the data yields an odds ratio for death with rt-PA versus the nonfibrin-specific agents of 0.59 (95% CI: 0.41 to 0.87), P = .0067. It should be stressed, however, that these results are derived from small studies that, although randomized, were not prospectively designed for mortality endpoints, and that final proof will need to be obtained from proper prospective randomized studies using conjunctive intravenous heparin with rt-PA.

APPRAOCHES TO IMPROVE THROMBOLYTIC THERAPY

Despite their widespread use, the currently available thrombolytic agents suffer from a number of significant limitations. Resistance to reperfusion occurs in 25% of patients despite the use of the most potent thrombolytic agents or combinations.3,4,6 Stable coronary patency is not uniformly produced,4,3,7,8 while angiographically documented acute coronary reocclusion occurs in 5% to 25% of patients.8,8 The time to reperfusion is prolonged, with restoration of anterograde coronary flow requiring on average 45 minutes after initiation of therapy.9,9 Significant bleeding may occur, with a frequency of intracerebral bleeding of approximately 0.5%.5,2 Both rt-PA and rscu-PA have a very short plasma half-life as a result of rapid hepatic clearance.10,11 Their therapeutic use probably requires continuous intravenous infusion, of relatively large amounts of material (50 to 100 mg), which is expensive. Therefore, the quest for, on the one hand, thrombolytic agents with a higher thrombolytic potency, specific thrombolytic activity, and/or a better fibrin selectivity and, on the other hand, for thrombolytic strategies that overcome resistance to clot lysis, accelerate recanalization, prevent reocclusion, and reduce the bleeding tendency, goes on.

Several lines of research towards improvement of thrombolytic agents are being explored, including the construction of mutants and variants of rt-PA or rscu-PA, chimeric (t-PA/u-PA) plasminogen activators, and conjugates or chimeras of plasminogen activators and monoclonal antibodies. In addition, the optimal mode and dose regimen of administration, the use of synergic combinations of thrombolytic agents, and the conjunctive use of antithrombotic agents need to be further explored.
Mutants and Variants of rt-PA

rt-PA mutants with deletion of the F, E, and/or K2 domains have a significantly reduced plasma clearance, which is, however, frequently associated with a reduced specific thrombolytic activity, resulting in an unchanged or only marginally improved thrombolytic potency. A variant of t-PA containing only the K2- and protease domain (K2P) was found to have a higher thrombolytic potency than rt-PA in a canine model of coronary artery thrombosis and a 20-fold reduced plasma clearance. Several variants of rt-PA, lacking the F-, E-, and/or K2-domains have been studied in a rabbit jugular vein thrombosis model. At an equivalent dose (on a weight base), all variants were less fibrin specific than t-PA; at a thrombotically equivalent dose, K2P caused a similar systemic fibrinolytic activation as t-PA; all variants had a reduced plasma clearance. However, in dogs with copper-coil-induced coronary artery thrombosis, K2P only had a marginally higher thrombolytic potency than t-PA after bolus injection. In aggregate, these studies suggest that domain deletion/substitution mutants of t-PA do not really constitute superior thrombolytic agents.

The t-PA of saliva from the vampire bat (Desmodus rotundus) is highly homologous with human t-PA, but lacks K2 and the plasmin cleavage site for conversion to a two-chain form. It was found to be stimulated 45,000-fold by fibrin and to constitute a potent and fibrin-specific thrombolytic agent in rabbits with femoral arterial thrombosis. In view of the large excess of t-PA over fibrin, the K2P-resistant mutants of t-PA may not constitute an obvious thrombolytic agent in rabbits with femoral arterial thrombosis.

Mutations in residues 296 to 304 of t-PA conferred resistance to PAI-1, with maintenance of the activity towards substrates. In view of the large excess of t-PA over PAI-1 achieved during thrombolytic therapy, resistance of rt-PA mutants to PAI-1 may not constitute an obvious advantage over wild-type t-PA. In as far as high PAI-1 levels may contribute to the occurrence of reocclusion, PAI-1-resistant mutants of t-PA may, however, be useful for maintenance infusion after initial thrombolysis.

Mutants and Variants of scu-PA

A low M, derivative of human scu-PA, scu-PA-32k, lacking the NH2-terminal 143 residues, was purified from cell cultures or was obtained by recombinant DNA technology. In a rabbit jugular vein thrombosis model, comparable clot lysis was obtained with scu-PA-32k and with M, 33,000 tcu-PA, and clot lysis with scu-PA-32k was associated with less pronounced systemic fibrinogen breakdown. scu-PA-32k may thus represent a useful alternative for large-scale production of a single-chain u-PA species by recombinant DNA technology. Deletion of the amino acid sequence Arg-179-Ser-184 in u-PA (which is homologous to the PAI-1 binding site of t-PA) was found to result in a urokinase mutant that is resistant to inhibition by PAI-1.

Chimeric (t-PA/u-PA) PAs

The rationale for the construction of chimeras between t-PA and u-PA (scu-PA or tcu-PA) is based on two observations. Firstly, the structures in t-PA responsible for its fibrin affinity are localized in the NH2-terminal region. Secondly, the fibrin specificity of scu-PA is not dependent on the NH2-terminal 143 amino acids, but is only preserved if the Lys-16-Ile-19 peptide bond is intact. Chimeric proteins consisting of parts of the NH2-terminal chain of t-PA and of the COOH-terminal region of scu-PA might thus combine the mechanisms of fibrin selectivity of both molecules.

Several chimeric PAs consisting of various portions of t-PA and u-PA have been constructed and partially characterized. Most investigators have obtained chimeras that have maintained the enzymatic properties of u-PA or of t-PA, confirming that the catalytic domains of both enzymes are functionally autonomous. The fibrin affinity of these chimeras is, however, usually lower than that of wild-type t-PA. The thrombolytic properties and fibrin specificity of such chimeras was found to be very similar but not superior to those of scu-PA. Combination of a partial fibrin affinity of t-PA with the enzymatic properties of scu-PA thus did not materially improve the thrombolytic potency of the molecule. Surprisingly, a chimera consisting of the two kringle domains of t-PA and the serine protease domain of scu-PA, which has little fibrin affinity in vitro, but has a 10-fold delayed in vivo clearance with relatively maintained specific thrombolytic activity, was found to have a markedly enhanced thrombolytic potency in venous and arterial thrombosis models in animals.

Complexes Between Monoclonal Antibodies and PAs

Murine monoclonal antibodies conjugated to PAs may be used for the targeting of the therapeutic agent to a thrombus. A thrombus contains both fibrin-rich and platelet-rich material, and targeting of plasminogen activators may thus be achieved via either antifibrin or antiplatelet monoclonal antibodies.

Anti-fibrin antibodies. Chemical conjugates of tcu-PA or scu-PA with monoclonal antibodies directed against the NH2-terminal of the Bβ-chain of fibrin or against fragment D-dimer of human cross-linked fibrin (MA-15CS) were shown to have an enhanced fibrinolytic potency in vitro. The conjugates had a fivefold to 10-fold higher thrombolytic potency and slower clearance than unconjugated scu-PA in experimental animal models of thrombosis.

Antibody targeting with fibrin-specific monoclonal antibodies thus appears to have the potential to increase the concentration of PA in the vicinity of a thrombus, thereby leading to enhanced clot lysis. The use of conjugates of PAs with specific monoclonal antibodies, prepared either chemically or by recombinant DNA technology, may allow a significant reduction of the total dose of PA with, hopefully, reduced systemic side effects.

Antiplatelet antibodies. Antiplatelet strategies may constitute important adjunctive treatments in thrombolysis and platelets may be used as a target for PAs. Bode et al. have chemically coupled tcu-PA to a monoclonal antibody that selectively binds to platelet membrane glycoprotein IIb/IIIa. This conjugate indeed targeted urokinase to the site of a platelet-rich clot, resulting in markedly enhanced clot lysis in human plasma in vitro. Dewerchin et al. have
prepared chemical conjugates of scu-PA with monoclonal antiplatelet antibodies, directed against thrombospondin or against ligand-induced binding sites on platelet glycoprotein IIb/IIIa. Such conjugates had a significantly enhanced thrombolytic potency towards platelet-rich clots in a hamster pulmonary embolism model.

**Conjointive Antithrombotic Therapy**

The efficacy of both aspirin and heparin to accelerate coronary thrombolysis, to overcome resistance to lysis, and to prevent reocclusion seems to be limited. Even with the concomitant administration of heparin and aspirin, thrombolytic therapy does not produce maximal stable coronary artery recanalization in patients with evolving myocardial infarction. The conjointive use of more potent and more selective antiplatelet and anticoagulant agents with PAs might constitute improved thrombolytic strategies.

Several alternative approaches to reduce platelet aggregation via pathways other than cyclooxygenase inhibition have recently been explored in animal models. These approaches include the use of thromboxane synthase inhibitors, antagonists of the serotonin or endoperoxide receptors, and platelet glycoprotein IIb/IIIa receptor-blocking agents. The latter group includes monoclonal antibodies directed against the platelet glycoprotein IIb/IIIa receptor, arginine-glycine-aspartic acid (RGD)-containing peptides derived from viper venoms, and small synthetic RGD-containing peptides. Another promising approach for the prevention of platelet-rich coronary artery thrombosis and for the acceleration of clot lysis consists of selective thrombin inhibition, with hirudin and its derivatives, with selective tripeptide chloromethyl ketones, or with synthetic thrombin inhibitors. Representatives from each of these groups of agents have been shown to be more effective than aspirin and/or heparin in preventing arterial thrombosis, in overcoming the resistance of platelet-rich thrombus to dispersion with thrombolytic agents, in accelerating arterial recanalization, and in reducing early and delayed reocclusion after reflow. However, several but not all of these combinations produce a significant prolongation of the bleeding time, which may be indicative of an increased bleeding risk.

**ACKNOWLEDGMENT**

D.C. is a party to a royalty-bearing licensing agreement for human tissue-type plasminogen activator between the University of Leuven, Leuven, Belgium, and Genentech, Inc, South San Francisco, CA, which could be perceived as a conflict of interest.

**REFERENCES**

61. Juhan-Vague I, Moerman B, De Cock F, Aillaud MF, Collen D: Plasma levels of a specific inhibitor of tissue-type plasminogen
 activator (and urokinase) in normal and pathological conditions. Thromb Res 33:523, 1984
64. United States Department of Health and Human Services: DHHS Publication No (PHS)85-1232. Hyattsville, MD, National Center for Health Statistics, 1984
68. Braunwald E: Myocardial reperfusion, limitation of infarct size, reduction of left ventricular dysfunction, and improved survival. Should the paradigm be expanded? Circulation 79:441, 1989
73. ISIS-3: Reported at a press conference preceding the American College of Cardiology meeting in Atlanta, March 2, 1991 (ISIS-3 press release and preliminary report)
78. Sherry SS, Marder VJ: Streptokinase and recombinant tissue plasminogen activator (rt-PA) are equally effective in treating acute myocardial infarction. Ann Intern Med 114:417, 1991
83. Neuhaus K: The t-PA versus APSAC patency study (TAPS). Presented at the American college of cardiology meeting, Atlanta, GA, 1991


98. Shebuski RJ, Fujita T, Ramjit DR: Thrombolytic efficacy of i.v. bolus vampire bat salivary plasminogen activator (tPA) in a rabbit model of femoral arterial thrombosis: Comparison to tissue-type plasminogen activator (t-PA). Fibrinolysis 4:97, 1990 (abstr 248, suppl 3).


Basic and clinical aspects of fibrinolysis and thrombolysis

D Collen and HR Lijnen