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COMMENTARY

MOLECULAR MECHANISMS OF THROMBOLYSIS: IMPLICATIONS FOR THERAPY

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Thrombotic complications of cardiovascular disease are a main cause of death and disability and, consequently, thrombolysis could favourably influence the outcome of such life-threatening diseases as myocardial infarction, cerebrovascular thrombosis, and venous thromboembolism.

Thrombolytic agents are plasminogen activators that convert plasminogen, the inactive proenzyme of the fibrinolytic system in blood, to the proteolytic enzyme plasmin. Plasmin dissolves the fibrin of a blood clot, but may also degrade normal components of the hemostatic system and predispose to bleeding. Currently, five thrombolytic agents are either approved for clinical use or under clinical investigation in patients with acute myocardial infarction. They are: streptokinase, urokinase, recombinant tissue-type plasminogen activator (rt-PA), anisoylated plasminogen streptokinase activator complex (APSAC), and single chain urokinase-type plasminogen activator (scu-PA, prourokinase). Streptokinase was the first thrombolytic agent used clinically, but it is only moderately efficacious and its administration is associated with extensive systemic fibrinogen breakdown. APSAC has a profile of thrombolytic efficacy and fibrin-specificity which is probably similar to that of streptokinase, but it can be administered by bolus injection. rt-PA is a more effective and fibrin-specific coronary arterial thrombolytic agent than streptokinase; scu-PA (prourokinase) is more fibrin-specific than urokinase but has only reached the stage of early clinical investigation.

Reduction of infarct size, preservation of ventricular function and/or reduction in mortality have been observed in patients with acute myocardial infarction treated with either streptokinase, rt-PA or APSAC. This review will deal with the molecular mechanism of action and with the thrombolytic properties of these three thrombolytic agents and with new developments in this area.

MECHANISM OF ACTION

Streptokinase

Streptokinase is produced by several strains of hemolytic streptococci; it consists of a single polypeptide chain with M_r 47,000–50,000 and contains 414 amino acids. The region comprising amino acids 1 to 230 shows some homology with trypsin-like serine proteinases but lacks an active site serine residue. Thus, streptokinase cannot directly cleave peptide bonds. Streptokinase activates plasminogen to plasmin indirectly, following a three-step mechanism. In the first step, streptokinase forms an equimolar complex with plasminogen, which 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 the third step, plasminogen–streptokinase molecules are converted to plasmin–streptokinase complex (for references, cf. Ref. 1).

Streptokinase disappears from the circulation with a half-life of approximately 20 min. The level of anti-streptokinase antibodies, which may result from previous infections with β-hemolytic streptococci, varies widely amongst individuals. Since streptokinase is inactivated by interaction with these antibodies, sufficient streptokinase must be infused to neutralize the antibodies. A few days after streptokinase administration, the anti-streptokinase titer rises rapidly to 50–100 times the preinfusion value and remains high for 4–6 months, during which period renewed treatment is impractical.

Anisoylated plasminogen streptokinase activator complex

APSAC is an equimolar non-covalent complex between human plasminogen and streptokinase. It has a catalytic centre located in the COOH-terminal region of the molecule, whereas the lysine-binding sites are found within the NH_2-terminal region of plasminogen. Thus, reversible acylation of the catalytic centre would not affect the fibrin-binding capacity of the complex [2]. The plasmin(ogen)–streptokinase complex is an efficient activator of plasminogen. APSAC binds to fibrin via the lysine-binding sites of plasminogen, although the affinity of plasminogen for fibrin is very weak. Deacylation of APSAC uncovers the catalytic centre, which converts plasminogen to plasmin. Deacylation of APSAC, however, does occur both in the circulation and at the fibrin surface.

Streptokinase slowly dissociates from the plasminogen–streptokinase complex with a rate constant

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of less than 10^{-4} sec^{-1}. The deacylation rate constant of APSAC, on the other hand, is greater than 10^{-4} sec^{-1}, which means that the activity of the complex will be controlled by the deacylation rate rather than by dissociation. In healthy volunteers, an apparent half-life of 70 min was found for APSAC, as compared to 25 min for the plasminogen–streptokinase complex formed upon administration of streptokinase. Patients with high streptokinase antibody titers (≥100 I.U./mL) showed very little response to low doses of APSAC. In some healthy volunteers, APSAC (12.5 units i.v. over 15–30 min) caused up to a 60-fold increase in the streptokinase antibody titer after 2–3 weeks, which was still high after 3 months (for references, cf. Ref. 3).

Tissue-type plasminogen activator

Tissue-type plasminogen activator (t-PA) is a serine proteinase with a M_r of about 70,000, originally isolated as a single polypeptide chain of 527 amino acids with Ser as the NH_2-terminal amino acid [4]. t-PA is converted by plasmin to a two chain form by hydrolysis of the Arg^{275}-Ile^{276} peptide bond. The NH_2-terminal region is composed of several domains with homologies to other proteins: residues 1–43 are homologous to the "finger domains" in fibronectin, residues 44–91 are homologous to human epidermal growth factor, and residues 92–173 and 180–261 are both homologous to the "kringle" regions of plasminogen. The region comprising residues 276–527 is homologous to that of other serine proteinases and contains the catalytic site, which is composed of His^{322}, Asp^{371}, and Ser^{478}. It was shown subsequently that native t-PA contains an NH_2-terminal extension of three amino acids (Gly-Ala-Arg) and that the correct numbering system should be based on a total of 530 amino acids (for references, cf. Ref. 5). Binding of t-PA to fibrin is mediated via the finger and second kringle domain. Kinetic data [6] support a mechanism in which fibrin provides a surface to which t-PA and plasminogen adsorb in a sequential and ordered fashion, yielding a cyclic 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 (low K_m) in the presence of fibrin thus allows efficient activation on the fibrin clot, whereas plasminogen activation by t-PA in plasma is a comparatively inefficient process (K_m three orders of magnitude higher). Plasmin formed on the fibrin surface has both its lysine-binding sites and active site occupied and is thus only slowly inactivated by α_2-antiplasmin (half-life of about 10–100 sec); in contrast, free plasmin, when formed, is rapidly inhibited by α_2-antiplasmin (half-life of about 0.1 sec). Early fibrin digestion by plasmin may result in acceleration of fibrinolysis by increasing the binding of both t-PA and plasminogen. Fibrinolysis by t-PA thus seems to be triggered by and confined to fibrin (for references, cf. Ref. 5).

In vivo experiments have indicated that t-PA has a half-life of only a few minutes and is eliminated almost exclusively by the liver, mainly via hepatocytes. Binding of t-PA to endothelial cells may also play a role in the removal of t-PA from the circulation *in vivo*; two different recognition systems for removal of t-PA by hepatocytes and by endothelial cells in the liver were characterized. For Activase® (predominantly single chain rt-PA, produced by suspension culture methods), the initial $T_{1/2}$ in patients with acute myocardial infarction was found to be 4 min and the terminal $T_{1/2}$ about 46 min. Evidence obtained with deletion mutants suggests that the structures involved in the rapid hepatic clearance of t-PA are localized in the NH_2-terminal region, comprising the finger and growth factor domains, but do, at present, not allow a more precise identification of the involved structures within this region (for references, cf. Ref. 5).

IMPLICATIONS FOR THERAPY

Reduction of infarct size, preservation of ventricular function and/or reduction in mortality have been observed with streptokinase, rt-PA and APSAC. Therefore, thrombolytic therapy will probably become routine therapy for early acute myocardial infarction. In patients with acute myocardial infarction, intravenous streptokinase recanalizes 40–45% of occluded coronary arteries and reduces mortality by 25%; it costs approximately $200 for a therapeutic dose of 1,500,000 units. Recombinant tissue-type plasminogen activator (rt-PA) is more potent for coronary arterial thrombolysis, producing both more rapid and more frequent (65–70%) reperfusion, but it costs $1,000 to over $2,000 for a therapeutic dose of 100 mg. Side-effects (mainly bleeding) and the incidence of reocclusion associated with the use of streptokinase and rt-PA are not markedly different. Although one could, on the basis of the significant correlation between coronary artery patency and clinical outcome, anticipate that the higher efficacy of rt-PA will translate into a comparably larger reduction of mortality, this remains to be confirmed in large comparative clinical trials. Both agents are available for clinical use. The choice of agent for the treatment of acute myocardial infarction at present must be based on considerations of lower cost of streptokinase versus the higher efficacy for coronary recanalization of rt-PA.

Thrombolytic therapy has been shown to reduce major deep vein thrombosis, but the impact on the subsequent development of the postphlebitic syndrome is not fully established. Thrombolytic therapy combined with heparin is recommended for patients with acute major pulmonary embolism who are hemodynamically unstable, whereas heparin alone is indicated in low risk patients. There is renewed interest in the treatment of peripheral arterial thromboembolic occlusion by local infusion of thrombolytic drugs. Thrombolytic treatment following a major ischemic stroke is hazardous although clinical improvement has been noted in a minority of patients with recanalized cerebral arteries. The safety and efficacy of thrombolytic treatment for this indication remain unproven, and its use must remain restricted to experimental protocols. Thrombolytic therapy in acute myocardial infarction, which presently constitutes the main indication for thrombolytic therapy, will be reviewed below.

Acute myocardial infarction

The recognition that thrombosis within the infarct

related coronary artery plays a major role in the pathogenesis of acute myocardial infarction [7], and the observation that early administration of either intracoronary or intravenous streptokinase results in reperfusion of 75 and 45% of patients, respectively [8], have formed the basis for several large scale studies with thrombolytic agents in acute myocardial infarction. These clinical trials with intravenous thrombolytics were designed to: (1) define the real impact of early coronary artery reperfusion on patient survival and (2) establish patterns of efficacy and safety for new and potentially improved thrombolytic agents.

Placebo-controlled trials with mortality end-points. The results of the major placebo-controlled clinical studies with intravenous thrombolytic therapy in patients with acute myocardial infarction are summarized in Table 1.

(a) *Streptokinase.* The GISSI study in early acute myocardial infarction was the first trial with intravenous streptokinase to demonstrate relative improvement in short-term and 1-year survival; 14% of the patients in this study also took aspirin. Life-threatening bleeding was infrequent (0.3%). The positive results on survival of the GISSI trial were not confirmed in a very similar but somewhat smaller study of the ISAM study group. Furthermore, in the ISAM-study, life-threatening bleeding was more common, occurring in 0.8% of patients, but all patients were also on aspirin. However, the ISIS-2 study has confirmed and extended the GISSI findings in 17,189 patients with an in-hospital mortality reduction from 11.7 to 8.9% in patients receiving streptokinase. This study also showed a significant reduction of mortality with aspirin alone and that the effects of streptokinase and aspirin are additive. Two additional studies, the Western Washington and New Zealand studies, have provided further evidence of the effect of streptokinase therapy on short-term or long-term mortality reduction and on its relative safety.

In aggregate, these studies are suggestive of two conclusions. First, the intravenous administration of streptokinase early (preferably within 4 hr) in acute myocardial infarction is of benefit to both early and late patient survival, producing a reduction in mortality of the order of 25%. Second, the incidence of life-threatening side-effects, predominantly major bleeding and intracranial hemorrhage, is not negligible but is not a major obstacle to thrombolytic therapy.

(b) *Tissue-type plasminogen activator.* Following the demonstration of the potential of t-PA as a thrombolytic agent and its production by recombinant DNA technology [4], improved left ventricular function and reduced mortality were demonstrated in placebo-controlled trials (Table 1).

Initial evaluation of rt-PA in myocardial infarction has been promising, suggesting its potential role as an improved thrombolytic agent [9, 10]. However, its use under present administration schemes is not risk-free, despite its markedly higher clot-selectivity than streptokinase.

(c) *Acylated plasminogen streptokinase activator complex.* In controlled studies comparing intravenous APSAC with intravenous or intracoronary streptokinase, APSAC was found to cause reperfusion at a frequency intermediate between that of intravenous and intracoronary streptokinase [11, 12]. A marked reduction in mortality with APSAC was published in a preliminary report of the AIMS Study [13].

Comparative biological properties of thrombolytic agents. At present, insufficient results of comparative clinical trials are available to allow definitive quantitative comparison of the relative efficacy and safety of the available thrombolytic agents. Sufficient information, however, is available from randomized controlled trials or from uncontrolled trials with comparable endpoints to allow a semi-quantitative comparison of rt-PA with streptokinase and of APSAC with streptokinase. The comparative properties of these thrombolytic agents, to the extent that they can at present be evaluated, are summarized in Table 2, and will be further detailed below.

(a) *Efficacy for coronary thrombolysis.* The efficacy of plasminogen activators for coronary thrombolysis in patients with acute myocardial infarction has been established by coronary angiography. Two randomized trials have been reported directly comparing the efficacy of streptokinase and rt-PA by angiography 90 min after the start of the infusion, namely the TIMI-I trial [14] and the first trial of the European Cooperative Study Group (ECSG) [15]. The TIMI-I trial was a reperfusion trial, whereas the ECGS trial was a patency trial. However, patency data from the TIMI-I trial have also been reported, recently, allowing pooling of the patency results of these two comparative trials [16]. The coronary patency at 90 min after the start of the infusion was 46% with streptokinase and 70% with rt-PA ($P < 0.001$).

The relative efficacy for coronary thrombolysis of acylated plasminogen streptokinase activator complex has been determined in controlled comparative trials with both intravenous streptokinase [11] and with intracoronary streptokinase [12]. Brochier *et al.* [11] determined angiographic patency at a mean of 105 min after intravenous administration of the acylated complex or intravenous streptokinase and found patency frequencies of 72% in 42 patients treated with the complex and of 56% in 44 patients given intravenous streptokinase, a difference that was not statistically significant. Anderson *et al.* [12] measured reperfusion rates in patients with proven coronary artery occlusion treated with intravenous acylated plasminogen streptokinase activator complex or intracoronary streptokinase. When the authors compared reperfusion with the complex at 90 min with that obtained with intracoronary streptokinase at 60 min, the reperfusion rates were 51 and 60%, respectively, a non-significant difference. The cumulative reperfusion rate with intracoronary streptokinase, however, was markedly higher than that with the complex at any given time point after the onset of therapy. In aggregate, these comparative studies suggest that the efficacy for coronary thrombolysis of acylated plasminogen streptokinase activator complex is comparable or somewhat higher than that of intravenous streptokinase, but lower than that of intracoronary streptokinase.

(b) *Bleeding complications.* Bleeding complications in association with thrombolytic therapy are

Table 1. Major placebo-controlled clinical trials with intravenous thrombolytic therapy in acute myocardial infarction

Study*	Number of patients	Adjunctive therapy		Major bleeding (%)		Neurological event rate (%)			Early mortality rate (%)			
		Aspirin	Heparin	Placebo	Active	Definition†	Placebo	Active	Endpoint (days)	Placebo	Active	P
(A) Streptokinase												
GISSI	11,806	14%	21%	?	0.3	Stroke	0.9	1.1	21	13.0	10.7	0.0002
						SK-CVA		0.2				
ISAM	1,741	+	+	?	0.8	ICB	0	0.5	21	7.1	6.3	NS‡
Western Washington	368	?	+	(0.7)	(13)	ICB	0	0.5	14	9.7	6.3	NS
New Zealand	219	+	+	0	1	ICB	0	0	30	12.9	2.5	0.012
ISIS-2	17,189	+/−	−	0.2	0.6	Stroke	0.9	0.8	35	11.7	8.9	0.0001
(B) rt-PA						SK-ICB		0.1–0.2				
ECSG-5	721	+	+	1.9	3.7	Stroke	0.5	2.0	14	5.7	2.8	0.053
						rt-PA-ICB		1.4				
ASSET	5,111	−	+ (24 hr)	0.4	1.4	Stroke	1.0	1.1	30	9.8	7.2	0.002
(C) APSAC												
AIMS	1,004	?	+	?	?	Stroke	1.0	0.4	30	12.2	6.4	0.0016

* References to these studies can be obtained elsewhere [9].

† Abbreviations: ICB, intracerebral bleeding; CVA, cerebrovascular accident; SK-CVA, CVA associated with streptokinase therapy; SK-ICB, ICB associated with SK therapy; and rt-PA-ICB, ICB associated with rt-PA therapy.

‡ Not significant.

+: present; −: absent.

Table 2. Comparative properties of thrombolytic agents in acute myocardial infarction*

	Streptokinase	APSAC	t-PA
Coronary thrombolysis			
Reperfusion	+ (43%)	+ or ++	+++ (69%)
Patency	+ (52%)	++	+++ (75%)
Speed of reperfusion	+	+ or ++	++
Reocclusion	++ (15%)	+ or ++	++ (15%)
Fibrin-specificity	−	−	++
Bleeding complications	++	++	+ or ++
Strokes†	+ (1%)	+	+ or ++ (?)
Allergic side-effects	+	+	−
Duration of infusion (min)	60	5	180
Dose (currently recommended or most frequently used)	1,500,000 units (~15 mg)	30 mg	100 mg
Early mortality reduction	+ (25%)	+ or ++	+ or ++

* At present there is insufficient data from comparative controlled trials with thrombolytic agents to allow definitive quantitative conclusions on their relative properties. In this table, an attempt is made to grade relative properties semiquantitatively (with +, ++, or +++) on the basis of results of controlled trials or of uncontrolled trials with sufficiently comparable design and endpoints. When sufficient data are available, quantitative estimates are included within brackets. Abbreviations: APSAC, acylated plasminogen streptokinase activator complex; and t-PA, tissue-type plasminogen activator.

† The stroke rate in the absence of thrombolytic therapy is approximately 1%.

most likely due to the combined actions of the thrombolytic agents on blood coagulation components, on the vessel wall and on the hemostatic plug. In addition, demographic characteristics of the patient and adjunctive anticoagulant or antiplatelet therapy may contribute to a bleeding tendency. Quantitative and qualitative evaluation of the bleeding incidence after treatment with different thrombolytic agents in non-comparative studies is very difficult, especially in association with highly variable frequencies of invasive cardiovascular procedures. Consequently, precise and detailed data on bleeding complications after treatment with different thrombolytic agents can only validly be compared in randomized controlled trials with these agents. In the four comparative trials with streptokinase and rt-PA, the frequency of bleeding complications was comparable in two trials [14, 17], and less frequent in the rt-PA group than in the comparative streptokinase group in the other two trials [15, 18]. Although presently available data from small randomized trials suggest a somewhat lower bleeding incidence with rt-PA as compared to streptokinase, the markedly higher fibrin-selectivity of rt-PA has not resulted in a proportional reduction of hemorrhagic complications.

In the comparative trials with APSAC and streptokinase, bleeding complication rates were found to vary widely, depending on the nature of the study (invasive or non-invasive). In the largest comparative study of APSAC with intravenous streptokinase [11], the bleeding complication rate was similar, whereas in the invasive study of Anderson *et al.* [12] bleeding and blood loss were more pronounced in the APSAC group than in the intracoronary streptokinase group.

(c) *Preservation of left ventricular function.* In several trials with thrombolytic agents, left ventricular function was measured by radionuclide or contrast ventriculography. Pooled results from trials assessing myocardial function after streptokinase administration have been presented using weighed means of ejection fraction [19]. A moderate but significant (2.9 to 6%) increase in ejection fraction assessed by radionuclide or contrast left ventricular angiography has been demonstrated in reperfused patients, while the others had a small deterioration in left ventricular function, measured in the first 72 hr and at the time of hospital discharge.

Significant improvements in ejection fraction have been demonstrated in patients with acute myocardial infarction in placebo-controlled trials with either rt-PA or with acylated plasminogen streptokinase activator complex (for references, cf. Refs. 3 and 20).

Although it is logical to assume that there is a cause–effect relationship between coronary artery reperfusion, preservation of left ventricular function, and reduction of early and late mortality, a review of the extensive experience with thrombolytic agents reveals that none of the placebo-controlled trials have demonstrated a simultaneous beneficial effect on both survival and global left ventricular function [21]. This paradox is further illustrated by a retrospective analysis of the data from the placebo-controlled rt-PA trial of the European Cooperative Study Group [22] which revealed that patients treated within 3 hr after the onset of symptoms had an 82% reduction in mortality at 14 days ($P = 0.009$) but no significant difference in ejection fraction (50.8% vs a control value of 49.8%), whereas patients treated after 3 hr had no significant reduction in mortality (4.5 and 5%) but a 4-point improvement in ejection fraction ($P < 0.01$) [21]. Van de Werf argued that administration of a thrombolytic agent early in the course of an acute myocardial infarct may save some patients with a very poor left ventricular function. The low ejection fractions of these surviving patients may mask the gain in ejection fraction obtained in other reperfused

patients, and distort the comparison with surviving patients in the control group.

Two studies have compared the effects of streptokinase and rt-PA on left ventricular function [17, 18]. White *et al.* [17] determined left ventricular function as the primary end-point in 270 patients with a first myocardial infarction treated within 3 hr of symptom onset. At 3 weeks, 10 patients had died in the streptokinase group and 5 patients in the rt-PA group (P = NS). Cineangiography revealed identical ejection fractions (58 ± 10%) in the survivors in both groups. Magnani *et al.* [18] studied 171 patients of which 7 patients treated with streptokinase and 4 patients treated with rt-PA died early. A significantly better ejection fraction was found in the rt-PA group as determined by echocardiography at discharge (56 vs 51%, P = 0.05) but not by contrast ventriculography within 4 days (55 vs 53%, P = NS).

Apart from the problems with distortion of results by differences in early mortality, these studies also suffer a problem of sample size. Indeed, all placebo-controlled studies with thrombolytic agents reported to date, in which left ventricular function was used as the primary endpoint, have entered patients in the control groups with global ejection fractions of 50 ± 10% and have reported improvement in the groups treated with thrombolytic agents in the order of 5 percentage points (to approximately 55 ± 10%). In such patient groups, a difference in efficacy between two agents of 50% would result in relative ejection fractions of 55 ± 10 and 57.5 ± 10% respectively. To demonstrate a significant difference in such studies, with a statistical power of 0.8 and an α value of 0.05, over 800 patients would have to be randomized. Consequently, more and larger studies are needed before one can make conclusive statements on the comparative effect of streptokinase and rt-PA on left ventricular function in patients with acute myocardial infarction.

(d) *Reduction of mortality.* The large variability in mortality in the control groups and the impact of thrombolytic agents thereon are influenced by patient selection, by adjunctive therapy including anticoagulant and antiplatelet drugs, and by mechanical coronary interventions. In this context, it is virtually impossible to compare the in-hospital mortalities of uncontrolled or placebo-controlled trials with the different thrombolytic agents in patients with acute myocardial infarction. Valid conclusions on the impact of thrombolytic agents will only be obtained from results of careful prospective, and large comparative trials, such as the ongoing GISSI-2 and the planned ISIS-3 trial. Similar restrictions hold for the interpretation of results obtained with APSAC. (See note added in proof.)

If the clinical benefit of thrombolytic therapy in patients with acute myocardial infarction is proportional to the efficacy of coronary thrombolysis, the size of randomized clinical trials required to establish differences between thrombolytic agents can be calculated. On the basis of controlled clinical trials of streptokinase versus placebo, in over 35,000 patients with acute myocardial infarction, the reduction of in-hospital mortality in the treatment group was found to be approximately 25%. Assuming that the impact on mortality is proportional to efficacy for coronary thrombolysis, a thrombolytic agent with a 50% higher efficacy than streptokinase would thus be anticipated to reduce early mortality by 37.5%. Assuming a control mortality of 9% in the absence of thrombolytic therapy, the mortality with streptokinase treatment would be reduced to 6.75% and that with the more potent agent to 5.6%. To establish such a difference with a statistical power of 0.8 and a significance level of 0.05, more than 10,000 patients would have to be entered into a randomized trial. These numbers illustrate the tremendous task to be undertaken in order to translate efficacy for coronary thrombolysis into the most relevant clinical endpoint, mortality.

In the meantime, it might well be of interest to review the available data from the four small comparative trials with streptokinase and rt-PA, carried out to date [14, 15, 17, 18]. Cumulative in-hospital mortalities were 34/443 (7.7%) in patients randomized to streptokinase and 24/442 (5.4%) in patients allocated to rt-PA [9]. It should be stressed, however, that these results are derived from small studies, albeit randomized, which were not prospectively designed for a mortality endpoint. However, they agree remarkably well with the values estimated on the basis of the hypothesis that the clinical outcome is determined primarily by the efficacy of the thrombolytic agent.

NEW STRATEGIES IN THE DEVELOPMENT OF THROMBOLYTIC AGENTS

Several strategies are being explored in order to improve the thrombolytic properties of presently available plasminogen activators. These lines of research include mutants of t-PA, chimeric plasminogen activators between urokinase-type plasminogen activator and t-PA, conjugates between fibrin-specific monoclonal antibodies and u-PA or t-PA, and synergic combinations of thrombolytic agents.

Mutants and variants of t-PA

Three main approaches have been followed for the development of t-PA mutants with a higher fibrin-affinity, a longer *in vivo* half-life, or an improved fibrin specificity. These include mutations at the plasmin cleavage site, deletion mutants of functional domains and glycosylation variants (for references, cf. Refs. 20 and 23).

Mutations of t-PA at the plasmin cleavage site. To study the functional consequences of the conversion of single chain t-PA to two chain t-PA, the Arg^{275} residue has been converted to glutamic acid by site-directed mutagenesis. The resulting mutant was found to have full plasminogen activating potential and stimulation by fibrin, without being converted to a two chain molecule. *In vivo*, in an arteriovenous shunt model in rabbits and dogs, mutant and wild type rt-PA were found to have a similar fibrinolytic activity. Subsequently, rt-PA molecules were constructed with each of the 20 amino acids at position 275. All mutants (except for Arg^{275} and Lys^{275}) remained in the single chain form and showed decreased plasminogen activating activity in the

absence of fibrin stimulation. The specific activities in the presence of fibrin, fibrin clot lysis activity and fibrin binding, however, were similar for all mutants except for Cys^{275}, which had comparatively less clot lysis and fibrin binding activity. Interestingly, all these mutants could be converted to a two chain molecule by plasmin (although only at a 1:10 ratio) by specific cleavage after Lys^{277}. This cleavage does not occur in the Arg^{275} and Lys^{275} mutants, presumably because prior hydrolysis at position 275 inhibits subsequent cleavage at Lys^{277}. A variant of t-PA in which Arg^{275} was replaced by Glu and Lys^{277} by Ile indeed remained in the single chain form and retained its ability to activate plasminogen in the presence of fibrin.

Deletion mutants of t-PA. The distinct domains in t-PA have been suggested to be involved in several functions of the enzyme including binding to fibrin, fast clearance *in vivo*, plasminogen activating activity with fibrin-specificity (enzymatic properties) and binding to endothelial cell receptors. Construction, expression and characterization of deletion mutants of t-PA, lacking one or more of these domains, has allowed a detailed study of structure–function relationships and investigation of the thrombolytic potential of such molecules.

Glycosylation variants of t-PA-ΔFE (deletion of F and E domains) were constructed, either with the glycosylated Asn^{117} residue mutagenized to Gln (t-PA-ΔFE1X), or with the three known glycosylated Asn residues replaced by Gln (t-PA-ΔFE3X). The specific thrombolytic activity and fibrin-specificity of these mutants in rabbits with jugular vein thrombosis were found to be comparable to those of natural t-PA. Upon bolus injection of t-PA-ΔFE3X in dogs with copper coil induced coronary artery thrombosis, this mutant was shown to be a more potent thrombolytic agent than t-PA.

Chimeric plasminogen activators

Chimeric t-PA/u-PA plasminogen activators. By comparing the properties of t-PA mutants obtained by deletion mutagenesis, van Zonneveld *et al.* [24] have suggested that the functional domains in t-PA are autonomous. In addition, the elucidation of the gene structure of t-PA has revealed that the domain pattern in the protein coincides with the intron/exon distribution in the gene. These findings have raised the potential to improve the fibrinolytic properties of plasminogen activators via the construction of recombinant chimeric proteins containing functional domains of t-PA and of other plasminogen activators. This hypothesis has mainly been evaluated for chimers between t-PA and u-PA, either in its single chain form (scu-PA) or its two chain form (tcu-PA).

The concept of the construction of chimeric molecules between t-PA and scu-PA is based on two observations. First, the structures in t-PA responsible for its fibrin-affinity are apparently localized in the A-chain (finger domain and second kringle). Second, the fibrin-specificity of scu-PA is not dependent on the NH_2-terminal 143 amino acids but is only preserved if the Lys^{158}-Ile^{159} peptide bond is intact. Chimeric proteins consisting of the A-chain of t-PA (amino acids Ser^{1} through Thr^{263}) and scu-PA-32k (amino acids Leu^{144} through Leu^{411}) might thus combine the mechanisms of fibrin-selectivity of both molecules. We have therefore constructed a hybrid human cDNA by splicing of a cDNA fragment of t-PA (encoding the A-chain) with a cDNA fragment of u-PA (encoding scu-PA-32k) and expressed the hybrid cDNA in CHO cells (t-PA/scu-PA-s). To minimize the steric interactions between the functional domains of t-PA and scu-PA, we have also constructed an extended chimer (t-PA/scu-PA-e) with an extension of 17 amino acids in the region joining the two proteins (Ser^{1} through Phe^{274} of t-PA and Ser^{138} through Leu^{411} of scu-PA). To avoid the presence of a free Cys in position 264 of t-PA, nucleotides 979 to 981 (TGC) were replaced by GGA which encodes Gly.

The functional properties (specific activity, catalytic efficiency for plasminogen activation) of these t-PA/u-PA chimeric plasminogen activators were found to be similar to those of u-PA (tcu-PA or scu-PA). Direct binding experiments of these chimeric molecules to fibrin revealed a significant fibrin-binding (about 50%) which was, however, lower than that of t-PA (about 90%).

In an *in vitro* fibrin clot lysis system, consisting of a ^{125}I-fibrin labeled plasma clot immersed in citrated human plasma, the chimeric molecules appeared to be equally effective and fibrin-specific as scu-PA but less effective than t-PA. The two chain chimers, like tcu-PA, appeared to be less fibrin-specific than the single chain molecules. The stability in plasma of the single chain chimers is comparable to that of scu-PA, whereas, the half-life time in plasma as well as the inhibition by α_2-antiplasmin of the two chain molecules is similar to that of tcu-PA. In rabbits, both the pharmacokinetic and thrombolytic properties of t-PA/scu-PA-s and t-PA/scu-PA-e are comparable to those of intact scu-PA.

In summary, the results obtained with chimeric t-PA/u-PA plasminogen activators indicate that it is possible to construct chimers which have maintained the enzymatic properties of u-PA (scu-PA or tcu-PA) or of t-PA and confirm that the catalytic domains of both t-PA and u-PA behave functionally autonomously. t-PA/u-PA chimers, however, have only about half of the fibrin affinity of wild-type t-PA. This may indicate that the fibrin binding domains in the NH_2-terminal region may not fold autonomously in t-PA/u-PA chimeric proteins. The effect of exon shuffling on the conformation and the function of chimeric proteins would therefore be unpredictable. Detailed knowledge of the tertiary structure of plasminogen activators would be required to resolve these problems.

Antibody-targeted thrombolytic therapy. Several alternatives to target the action of thrombolytic agents towards the thrombus with the use of fibrin-specific antibodies are being investigated presently. These include chemical conjugates of fibrin-specific antibodies with urokinase or rt-PA, or recombinant fusion proteins comprising a fibrin-specific antibody site and the β-chain of t-PA [25, 26].

Urokinase was linked covalently to a monoclonal antibody (64C5) specific for the aminoterminus of the β-chain of human fibrin, by means of the cross-linking reagent *N*-succinimidyl-3-(2-pyridyldithio)

propionate (SPDP) following introduction of sulfhydryl groups with 2-iminothiolane. Alternatively, the inter-heavy chain sulfhydryl of the Fab′ of the antibody was linked to SPDP-substituted urokinase. Both conjugates lysed fibrin monomer with an about 100-fold higher potency than urokinase, indicating that the antibody had maintained its fibrin-specificity and that univalent binding to fibrin (Fab′-urokinase) is sufficient to enhance fibrinolysis. The fibrinolytic potency of the Fab′-urokinase conjugate towards cross-linked thrombi in citrated plasma was found to be only 4.4 times that of uncoupled urokinase [27].

scu-PA was also conjugated to the Fab′ fragment of monoclonal antibody 59D8. In *in vitro* assays, the fibrinolytic potency of scu-PA-59D8 was 230-fold greater than that of rscu-PA, 420-fold greater than that of urokinase and 33-fold greater than that of t-PA. These findings suggest that conjugation to a fibrin-specific antibody confers fibrin affinity to scu-PA, while its functional and structural properties are maintained.

Schnee *et al.* [28] have engineered a recombinant version of the t-PA-59D8 conjugate. The 59D8 heavy chain gene was cloned and combined in an expression vector with sequence coding for a portion of the γ2b constant region and the β-chain of t-PA with the catalytic site. This construct was transfected into cloned cells derived from the 59D8 hybridoma which had lost their expression of the heavy chain. The hybrid proteins had antifibrin antibody activity and retained plasminogen activating potential.

Alternatively, chemical conjugates between a fibrin-specific and a t-PA-specific antibody, and biosynthetically produced heteroduplex antibodies that are both fibrin and t-PA specific, could bind to fibrin and localize endogenous or exogenous t-PA [25]. Addition of both the heteroduplex antibody and t-PA to a plasma clot lysis system resulted in significantly more lysis than observed with t-PA alone. It thus appears to be possible to concentrate t-PA (added separately) to the site of a fibrin clot in plasma using a heteroduplex antibody with specificities for both t-PA and fibrin.

We have constructed a chemical conjugate between scu-PA and a murine monoclonal antibody specific for fragment D-dimer of human cross-linked fibrin. In a rabbit jugular vein thrombosis model with the use of human thrombi *in vivo*, the specific thrombolytic potency of this conjugate was 8-fold higher than that of scu-PA due to specific targeting to the thrombus [29]. The plasma clearance rate of the conjugate was 4-fold slower than that of non-conjugated scu-PA. To prevent immunological reactions in humans, efforts are undertaken to "humanize" murine monoclonal antibodies by substituting their constant regions with corresponding structures derived from human antibodies.

Synergism of plasminogen activators

The intrinsic fibrin selectivity of t-PA and scu-PA is mediated by entirely different molecular mechanisms. It is therefore not unreasonable to expect that, if administered in combination, the effect on clot dissolution might be more than additive. Synergism, if significant, would allow a reduction of the total dose while obtaining a therapeutic effect that is equal to that achieved with higher doses of either agent alone (for references, cf. Ref. 30). As a result, the potential for hemostatic side-effects might be reduced significantly. *In vivo* in a jugular vein thrombosis model in the rabbit, significant synergism between t-PA and scu-PA and between t-PA and urokinase for thrombolysis was observed [31]. When t-PA and scu-PA were infused in a molar ratio of approximately 1:3, the specific thrombolytic activity of the mixture was approximately 3-fold higher than was anticipated on the basis of additive effects of both agents. The synergistic effect of t-PA and urokinase was only of borderline significance.

Preliminary results in patients with acute myocardial infarction [32, 33] suggest that t-PA acts synergistically with scu-PA and urokinase in humans as well. Indeed, combining t-PA and scu-PA at approximately one-fifth of their individual thrombolytic doses produced coronary artery reperfusion in patients with acute myocardial infarction without associated systemic fibrinogen breakdown. Although these results are still preliminary and need to be confirmed in larger studies, they are potentially of significant clinical importance. The synergistic effect of t-PA and urokinase on coronary reperfusion, however, has not been confirmed in a large-scale study, although the use of the combination was associated with a significant reduction of the frequency of reocclusion [34].

CONCLUSIONS

Thrombotic complications of cardiovascular diseases are a main cause of death and disability. Consequently, one might assume that thrombolysis could favorably influence the outcome of such life-threatening diseases as myocardial infarction, cerebrovascular thrombosis, and venous thromboembolism.

Although streptokinase was first administered to humans over 30 years ago, it was not widely used, mainly because of uncertainty about the optimal dose, fear of bleeding complications, and a poor correlation between the results of laboratory parameters and the incidence of either therapeutic success or of bleeding complications. Interest in thrombolytic therapy has been renewed by the demonstration that acute myocardial infarction is caused most frequently by thrombotic occlusion of an atherosclerotic coronary artery and that the early administration of streptokinase significantly reduces mortality. The efficacy of streptokinase, however, is limited and rapidly decreases with time after the onset of coronary artery thrombosis. In addition, it has no fibrin selectivity and its use is associated with extensive breakdown of the hemostatic system; yet the occurrence of life-threatening bleeding in association with high-dose, short-duration therapy is consistently less than 1%.

A better understanding of the molecular mechanisms that regulate fibrinolysis *in vivo*, the identification of the physiological plasminogen activators t-PA and scu-PA with high degrees of fibrin selectivity, and the development of recombinant DNA technology allowing large-scale production of these proteins have each fueled the hope that newer and better thrombolytic drugs may become available. One of

the new generation of thrombolytic agents, t-PA, has now been widely studied. These studies indicate that it is more efficacious, more fibrin specific and, when used in adequate dose, is at least as safe as streptokinase. Important issues such as its impact on morbidity and mortality and its cost–benefit ratio remain to be resolved. The physiological plasminogen activators t-PA and scu-PA, however, still suffer significant shortcomings, including the need for large doses to be therapeutically efficient, a limited fibrin specificity, and a residual toxicity in terms of bleeding complications. Consequently, research into the development of still further improved therapeutic regimens or thrombolytic agents is intensifying.

Thus, thrombolytic therapy, despite a 30-year period of relatively slow development, has become routine treatment for early acute myocardial infarction and possibly for other thrombotic cardiovascular diseases. Further improvements of thrombolytic agents to maximize efficacy, minimize side-effects, and optimize cost–benefit ratios are needed. It is anticipated that such improvements will emerge from several of the new lines of ongoing preclinical and clinical research.

Note added in proof—The recent studies of Bleich *et al.* (*Circulation* **80**: 11–113, 1989) and of Ross *et al.* (unpublished, presented at the American Heart Association meeting, November 1989) have reported a significantly lower patency rate, determined by coronary angiography after a mean of 55–59 h and 7–24 h respectively, when rt-PA was administered without heparin in patients with acute myocardial infarction. The patency rates obtained with rt-PA in the absence of heparin were similar to those previously obtained with streptokinase in the presence of heparin. On the other hand it has been claimed that streptokinase, because of the profound hypocoagulable state which it produces in the blood stream for many hours, does not require adjuvant heparin therapy for coronary recanalization (Sherry, *J Am Coll Cardiol* **12**: 519–525, 1988). Thus it is most likely that the previously established higher reperfusion rates of rt-PA as compared to streptokinase requires the concomitant use of heparin with rt-PA, but probably not with streptokinase. Therefore, the GISSI-2 and ISIS-3 trials, because of their design (heparin started 12 h and 3 h after thrombolytic therapy respectively and administered by subcutaneous injection), cannot be expected to satisfactorily resolve the crucial question whether efficacy for coronary recanalization translates into clinical benefit. Indeed, with administration schemes that probably produce comparable rates of recanalization, clinical outcomes are expected to be comparable.

REFERENCES

1. Reddy KNN, Streptokinase—Biochemistry and clinical application. *Enzyme* **40**: 78–89, 1988.
2. Smith RAG, Dupe RJ, English PD and Green J, Fibrinolysis with acylenzymes: A new approach to thrombolytic therapy. *Nature* **290**; 505–508, 1981.
3. Monk JP and Heel RC, Anisoylated plasminogen streptokinase activator complex (APSAC). A review of its mechanism of action, clinical pharmacology and therapeutic use in acute myocardial infarction. *Drugs* **34**: 25–49, 1987.
4. Pennica D, Holmes WE, Kohr WJ, Harkins RN, Vehar GA, Ward CA, Bennett WF, Yelverton E, Seeburg PH, Heyneker HL, Goeddel DV and Collen D, Cloning and expression of human tissue-type plasminogen activator cDNA in *E. coli*. *Nature* **301**: 214–221, 1983.
5. Lijnen HR and Collen D, New strategies in the development of thrombolytic agents. *Blut* **57**: 147–162, 1988.
6. Hoylaerts M, Rijken DC, Lijnen HR and Collen D, Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. *J Biol Chem* **257**: 2912–2919, 1982.
7. De Wood MA, Spores J, Notske R, Lowell T and Mouser T, Prevalence of total coronary occlusion during the early hours of transmural myocardial infarction. *N Engl J Med* **303**: 897–902, 1980.
8. Rentrop KP, Thrombolytic therapy in patients with acute myocardial infarction. *Circulation* **71**: 627–631, 1985.
9. Collen D, Stump DC and Gold HK, Thrombolytic therapy. *Annu Rev Med* **39**: 405–423, 1988.
10. Collen D, Human tissue-type plasminogen activator: From the laboratory to the bedside. *Circulation* **72**: 18–20, 1985.
11. Brochier ML, Quilliet L, Kulbertus H, Matterne P, Letac B, Cribier A, Monassier JP, Sacrez A and Favier JP, Intravenous anisoylated plasminogen streptokinase activator complex versus intravenous streptokinase in evolving myocardial infarction. *Drugs* **33** (Suppl 3): 140–145, 1987.
12. Anderson JL, Rothbard RL, Hackworthy RA, Sorensen SG, Fitzpatrick PG, Dahl CF, Hagan AD, Browne KF, Symkoviak GP, Menlove RL, Barry WH, Eckerson HW and Marcher VJ, Multicenter reperfusion trial of intravenous anisoylated plasminogen streptokinase activator complex (APSAC) in acute myocardial infarction: Controlled comparison with intracoronary streptokinase. *J Am Coll Cardiol* **11**: 1153–1163, 1988.
13. AIMS Trial Study Group, Effect of intravenous APSAC on mortality after acute myocardial infarction: Preliminary report of a placebo-controlled clinical trial. *Lancet* **1**: 545–549, 1988.
14. The TIMI Study Group, Special Report. The thrombolysis in myocardial infarction (TIMI) trial. *N Engl J Med* **312**: 932–936, 1985.
15. Verstraete M, Bernard R, Bory M, Brower RW, Collen D, de Bono DP, Erbel R, Huhmann W, Lennane RJ, Lubsen J, Mathey D, Meyer J, Michels HR, Rutsch W, Schartl M, Schmidt W, Uebis R and von Essen R, Randomized trial of intravenous recombinant tissue-type plasminogen activator versus intravenous streptokinase in acute myocardial infarction. *Lancet* **1**: 842–847, 1985.
16. Chesebro J, Knatterud G and Braunwald E, Correspondence section (Letter). *N Engl J Med* **319**: 1544, 1988.
17. White HD, Rivers JT, Maslowski AH, Ormiston JA, Takayama M, Hart HH, Sharpe DN, Whitlock RML and Norris RM, Effect of intravenous streptokinase as compared with that of tissue plasminogen activator on left ventricular function after first myocardial infarction. *N Engl J Med* **320**: 817–821, 1989.
18. Magnani B, for the PAIMS Investigators, Plasminogen Activator Italian Multicenter Study (PAIMS). Comparison of intravenous recombinant single-chain human tissue-type plasminogen activator (rt-PA) with intravenous streptokinase in acute myocardial infarction. *J Am Coll Cardiol* **13**: 19–26, 1989.
19. Patel B and Kloner RA, A critical analysis of streptokinase therapy for acute myocardial infarction. *Circulation* **72**: III–412, 1985.
20. Collen D, Lijnen HR and Verstraete M, The fibrinolytic system and its disorders. In: *Blood: Principles and Practice of Hematology* (Eds. Handin RI, Lux SE and Stossel JP, JB Lippincott, Philadelphia, in press.

21. Van de Werf F. Discrepancies between the effects of coronary reperfusion on survival and left ventricular function. *Lancet* **1**: 1367–1368, 1989.
22. Van de Werf F and Arnold AER, Intravenous tissue plasminogen activator and size of infarct, left ventricular function, and survival in acute myocardial infarction. *Br Med J* **297**: 1374–1379, 1988.
23. Krause J, Catabolism of tissue-type plasminogen activator (t-PA), its variants, mutants and hybrids. *Fibrinolysis* **2**: 133–142, 1988.
24. van Zonneveld AJ, Veerman H and Pannekoek H, Autonomous functions of structural domains on human tissue-type plasminogen activator. *Proc Natl Acad Sci USA* **83**: 4670–4674, 1986.
25. Haber E, Runge M, Bode C, Branscomb B, Schnee J, Quertermous T and Matsueda G, Antibody targeted fibrinolysis. *Thromb Haemost* **57**: 253, 1987.
26. Haber E, Quertermous T, Matsueda GR and Runge MS, Innovative approaches to plasminogen activator therapy. *Science* **243**: 51–56, 1989.
27. Bode C, Runge MS, Newell JB, Matsueda GR and Haber E, Thrombolysis by a fibrin-specific antibody Fab'-urokinase conjugate. *J Mol Cell Cardiol* **19**: 335–341, 1987.
28. Schnee JM, Runge MS, Matsueda GR, Hudson NW, Seidman JC, Haber E and Quertermous T, Construction and expression of a recombinant antibody-targeted plasminogen activator. *Proc Natl Acad Sci USA* **84**: 6904–6908, 1987.
29. Collen D, Dewerchin M, Stassen JM, Lijnen HR and Nelles L, Thrombolytic and pharmaco-kinetic properties of conjugates of urokinase-type plasminogen activator with a monoclonal antibody specific for cross-linked fibrin. *Fibrinolysis* **3**: 197–202, 1989.
30. Collen D, Synergism of thrombolytic agents: Investigational procedures and clinical potential. *Circulation* **77**: 731–735, 1988.
31. Collen D, Stassen JM, Stump DC and Verstraete M, Synergism of thrombolytic agents *in vivo*. *Circulation* **74**: 838–842, 1987.
32. Collen D, Stump DC and Van de Werf F, Coronary thrombolysis in patients with acute myocardial infarction by intravenous infusion of synergic thrombolytic agents. *Am Heart J* **112**: 1083–1084, 1986.
33. Collen D and Van de Werf F, Coronary arterial thrombolysis with low-dose synergistic combinations of recombinant tissue-type plasminogen activator (rt-PA) and recombinant single-chain urokinase-type plasminogen activator (rscu-PA) for acute myocardial infarction. *Am J Cardiol* **60**: 431–434, 1987.
34. Topol EJ, Califf RM, George BS, Kereiakes DJ, Rothbaum D, Candela RJ, Abbottsmith CW, Pinkerton CA, Stump DC, Collen D, Lee KL, Pitt B, Keine EM, Boswick JM, O'Neill WW, Stack RS and the TAMI Study Group, Coronary arterial thrombolysis with combined infusion of recombinant tissue-type plasminogen activator and urokinase in patients with acute myocardial infarction. *Circulation* **77**: 1100–1107, 1988.