CORONARY REPERFUSION:

Beyond the Event Horizon

Internal Medicine Grand Rounds

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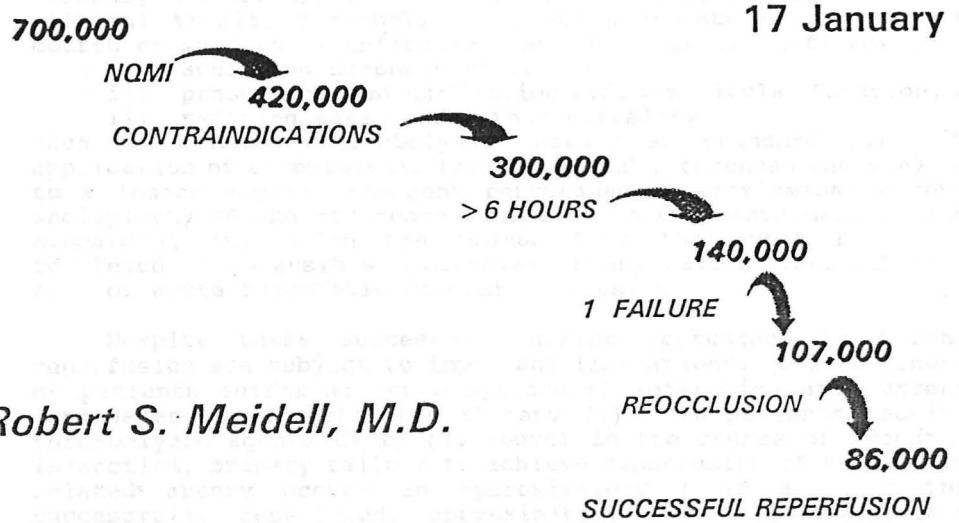

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INTRODUCTION

Thirty years ago, acute myocardial infarction was viewed as a discrete, immutable event. Over the next two decades, identification of the role of occlusive coronary thrombosis in the pathophysiology of "transmural" (Q-wave) myocardial infarction (1), and the perception of ischemic myocardial necrosis as a dynamic process occurring over several hours following coronary occlusion (2), opened a potential window of opportunity. Intervention to reestablish coronary perfusion early in the acute phase of myocardial infarction might salvage jeopardized myocardium and limit infarct size. Identification of residual post-infarction ventricular function as the most important determinant of survival following myocardial infarction resulted in early attempts to achieve emergent surgical coronary revascularization (3).

The field of Cardiology has been dominated in the last decade by the development of practical, effective strategies to achieve coronary reperfusion. In extensive prospective, randomized clinical trials, thrombolytic agents administered early in the course of myocardial infarction have demonstrated efficacy in:

i) achieving coronary reperfusion
ii) preserving post-infarction left ventricular function, and
iii) reducing early and 1 year mortality,

thus establishing thrombolytic therapy as standard (4). The application of thrombolytic therapy (5 and references therein), and to a lesser degree, emergent percutaneous transluminal coronary angioplasty (6 and references therein) to the management of acute myocardial infarction has pushed back the event horizon of completed, irreversible myocardial injury several hours from the time of acute thrombotic coronary occlusion.

Despite these successes, current approaches to coronary reperfusion are subject to important limitations. Only a minority of patients suffering acute myocardial infarction are currently candidates for thrombolytic therapy (7). In patients receiving thrombolytic agents early (<4 hours) in the course of myocardial infarction, primary failure to achieve reperfusion of the infarct-related artery occurs in approximately 1 in 4. Of those successfully reperfused, approximately 20% will suffer early thrombotic reocclussion and reinfarction, with an important attendant effect on early mortality (8). The time required to achieve coronary reperfusion with current approaches, generally 45 to 90 minutes, limits the degree of myocardial salvage, and may therefore limit the beneficial effects on survival (9).

In addition, recent clinical observations suggest that the current conceptual paradigm, that coronary reperfusion results in improved survival as a result of the limitation of infarct size, may be incomplete (10). A causal relationship between myocardial salvage and long term survival has not been clearly established, and some observations suggest that the currently defined event

horizon, roughly six hours following acute coronary occlusion, may be artifactual.

In today's discussion, I intend to:

i) review briefly clinical trials of coronary reperfusion in the setting of acute myocardial infarction, emphasizing observations which suggest that the currently defined window of opportunity for myocardial salvage may inappropriately limit the application of effective therapy;

ii) provide an overview of selected novel strategies, largely in stages of basic investigation, which offer some potential to reduce the rates of primary and/or secondary failure, and;

iii) examine potential limitations in the current doctrine governing the therapeutic application of thrombolytic agents, i.e., to examine the potential of coronary reperfusion beyond the event horizon.

CLINICAL CORONARY REPERFUSION

THROMBOLYTIC THERAPY

Coronary angiography performed in the acute phase of Q-wave myocardial infarction demonstrates complete thrombotic occlusion of an epicardial coronary artery in approximately 90% of patients (2). The efficacy of thrombolytic agents administered early (<6 hours) after the onset of symptoms, in reestablishing angiographic coronary patency and antegrade coronary blood flow has been evaluated in seven prospective, randomized clinical trials. The results of these studies are summarized in Figure 1. Available agents are effective in achieving patency of the infarct-related artery in approximately 70% of patients treated within 4 hours of the onset of symptoms. Analysis of pooled data suggest that human rt-PA is somewhat more effective than available alternative agents, streptokinase and acyl-plasminogen streptokinase activator complex (APSAC, anistreplase) (10), although this issue is debated. Approximately 25% of patients treated with these agents early in the course of myocardial infarction are primary therapeutic failures, failing to achieve effective coronary reperfusion.

Effective coronary reperfusion results in improved post-infarction global left ventricular function. Figure 2 summarizes the results of seven prospective clinical trials which have examined the effect of thrombolytic therapy on post-infarction left ventricular ejection fraction as a primary endpoint. Two observations are apparent;

i) in each of these trials, there is at least a trend toward improved global ventricular function in patients receiving thrombolytic therapy, and;

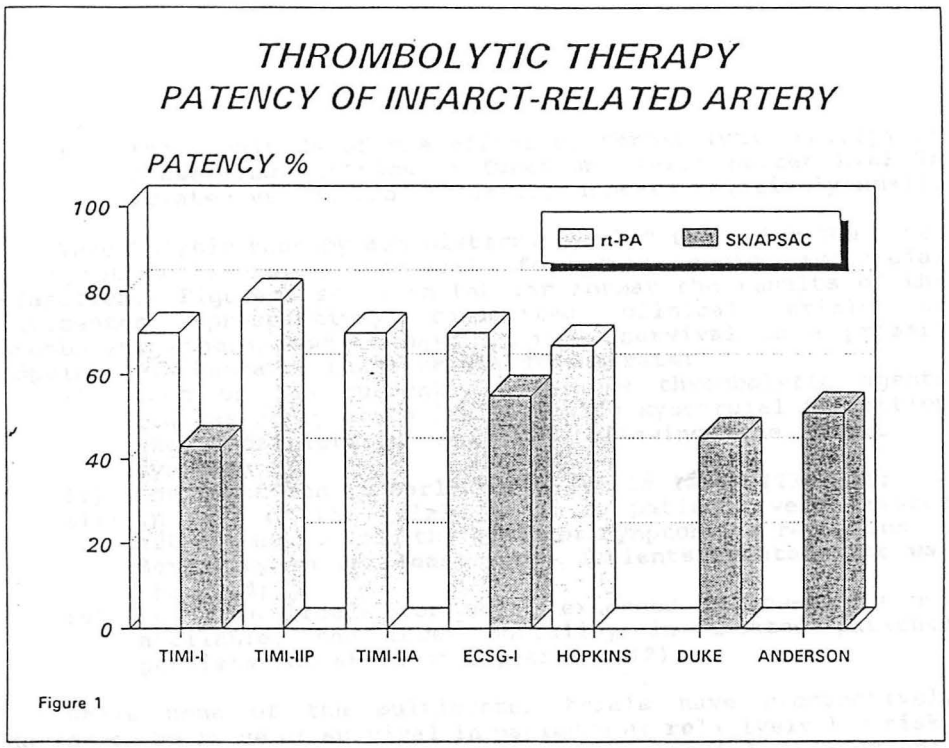


Figure 1

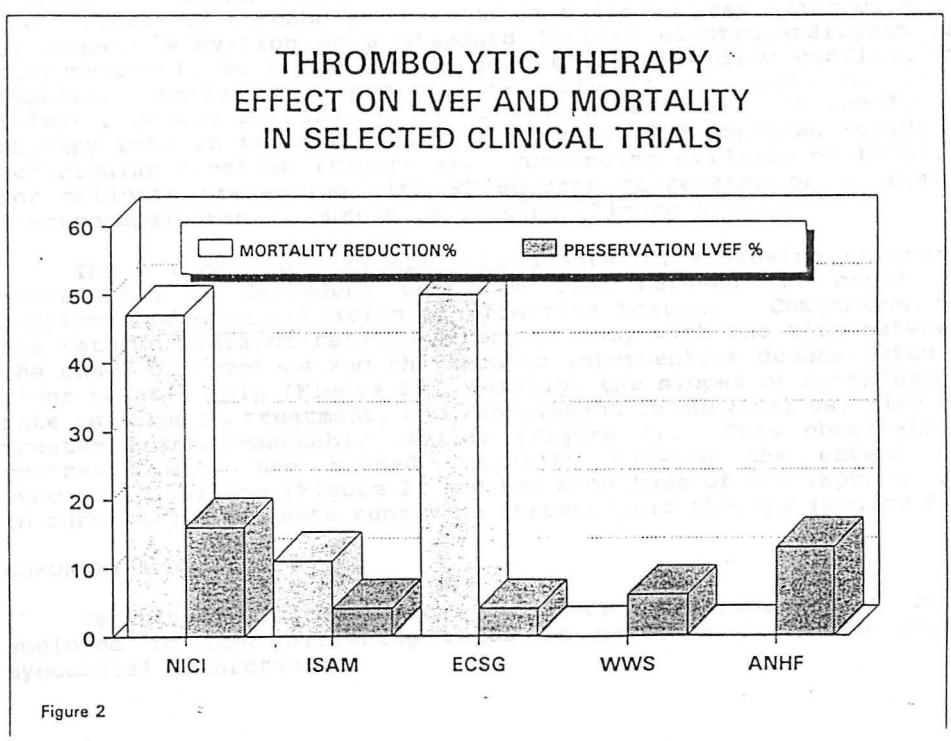


Figure 2

ii) the magnitude of the effect of thrombolytic therapy in preserving ventricular function (4-23% better LVEF in treated vs. control patients) appears relatively small.

Thrombolytic therapy administered "early" following the onset of symptoms improves survival following acute myocardial infarction. Figure 3 shows in tabular format the results of the multicenter, prospective, randomized clinical trials of thrombolytic therapy which have examined survival as a primary endpoint. In summary, these trials demonstrate:

i) each of the currently available thrombolytic agents reduces early mortality following myocardial infarction when administered shortly following the onset of symptoms;
ii) the reduction in early mortality is large (23-52%);
iii) in each of the trials in which patients were treated after 6 hours from the onset of symptoms, a reduction in mortality in (at least some) patients treated late was observed;
iv) in those trials for which extended follow-up is now available, the lower mortality in treated patients persists for at least 1 year (11,12).

While none of the multicenter trials have prospectively addressed the issue of survival in patients at relatively low risk, i.e. those with inferior or non Q-wave myocardial infarctions, extensive subgroup analysis of the available data is available. The efficacy of thrombolytic agents in patients presenting without ST segment elevation on a standard 12-lead electrocardiogram is controversial, with the two largest trials yielding conflicting results. Analysis of pooled data suggests that patients with inferior Q-wave myocardial infarctions benefit from thrombolytic therapy both in terms of improved survival and improved residual ventricular function (Figure 4). Convincing evidence of benefit for patients presenting with ST segment depression or a normal electrocardiogram is currently lacking (Figure 5).

The efficacy of thrombolytic agents in achieving coronary reperfusion is dependent upon the time between the onset of symptoms and administration of effective therapy. Comparison of the rates of infarct-related artery patency with the time between the onset of symptoms and therapeutic intervention demonstrates a clear relationship (Figure 6). Notably, the slopes of reperfusion rate vs. time to treatment, and improvement in survival vs. time to treatment are remarkably similar (Figure 7). This observation contrasts with the marked disparity between the extent of myocardial salvage (Figure 2) and the magnitude of the improvement in survival in patients receiving thrombolytic therapy (Figure 3).

CORONARY ANGIOPLASTY

Percutaneous transluminal coronary angioplasty has been employed in four differing roles in patients suffering acute myocardial infarction:

THROMBOLYSIS AND MORTALITY

TRIAL	N	AGENT	T	% Δ M
NICI	535	SK(IC)	6	22%
GISSI	11,806	SK	12	18%
ISAM	1741	SK	6	26%
ISIS-2	17,187	SK/ASA	24	25%/42%
ECSG	721	tPA	6	51%
ASSET	5031	tPA	5	26%
AIMS	992	APSAC	6	50%

Figure 3

THROMBOLYSIS IN IMI

TRIAL	MORTALITY %	
	CONTROL	TREATED
ECSG	20.6	11.6
ISAM	14.2	10.3
GISSI	7.2	6.8
ISIS-2	8.8	7.2
ASSET	9.8	6.3
AIMS	7.8	3.3

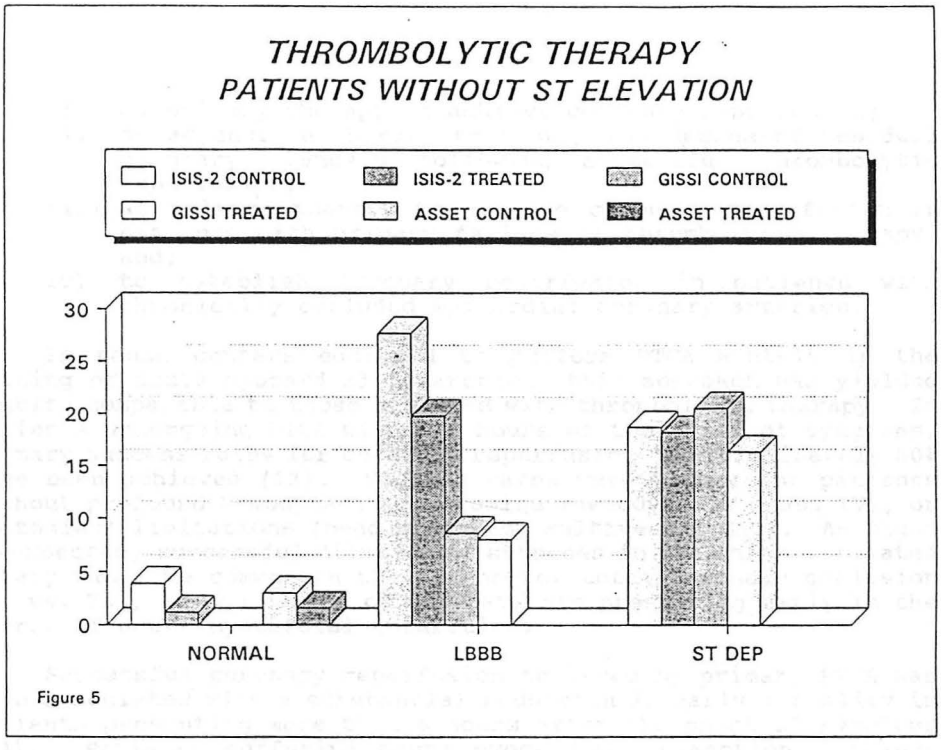


Figure 5

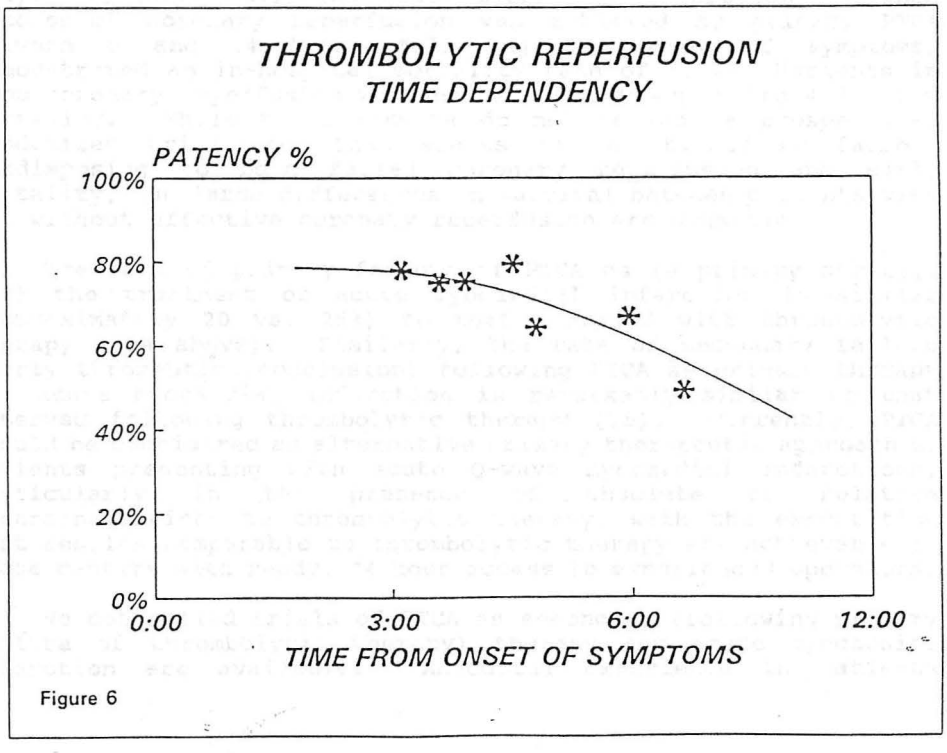


Figure 6

i) as primary therapy to achieve coronary reperfusion;
ii) as adjunctive therapy to reduce the degree of residual coronary stenosis following successful thrombolytic reperfusion;
iii) as salvage therapy to achieve coronary reperfusion in patients with primary failure of thrombolytic therapy, and;
iv) to establish coronary reperfusion in patients with chronically occluded epicardial coronary arteries.

In those centers equipped to perform PTCA acutely in the setting of acute myocardial infarction, this approach has yielded results comparable to those achieved with thrombolytic therapy. In patients undergoing PTCA within 6 hours of the onset of symptoms, primary success rates for coronary reperfusion of approximately 80% have been achieved (13). Success rates were higher for patients without profound hemodynamic compromise (hemodynamic class IV), or anatomical limitations (bend stenoses, multivessel CAD). As might be expected, successful dilation of stenoses in the infarct-related artery was more common in the absence of total coronary occlusion (85 vs. 78%) in one series of 384 patients presenting early in the course of acute myocardial infarction.

Successful coronary reperfusion achieved by primary PTCA has been associated with a substantial reduction in early mortality in patients presenting more than 6 hours after the onset of symptoms (14). Patients suffering acute myocardial infarction, in whom successful coronary reperfusion was achieved by primary PTCA between 6 and 24 hours following the onset of symptoms, demonstrated an in-hospital mortality rate of 5.5%. Patients in whom coronary reperfusion was unsuccessful demonstrated 43% early mortality. While these results do not reflect a prospective, randomized trial, and the results may be biased by factors predisposing to both failed coronary reperfusion and early mortality, the large differences in survival between patients with and without effective coronary reperfusion are dramatic.

The rate of primary failure of PTCA as (a primary strategy for) the treatment of acute myocardial infarction is similar (approximately 20 vs. 25%) to that observed with thrombolytic therapy (see above). Similarly, the rate of secondary failure (early thrombotic reocclusion) following PTCA as primary therapy for acute myocardial infarction is remarkably similar to that observed following thrombolytic therapy (15). Currently, PTCA should be considered an alternative primary therapeutic approach to patients presenting with acute Q-wave myocardial infarctions, particularly in the presence of absolute or relative contraindications to thrombolytic therapy, with the expectation that results comparable to thrombolytic therapy are achievable in those centers with ready, 24 hour access to experienced operators.

No controlled trials of PTCA as secondary (following primary failure of thrombolytic therapy) therapy for acute myocardial infarction are available. Anecdotal experience in patients

enrolled in the TIMI-II and TAMI-I trials, however, suggests that salvage PTCA may impact survival in patients failing thrombolytic therapy. In these studies, 112 patients underwent rescue PTCA following failed thrombolytic therapy. Successful reperfusion, judged angiographically, was achieved in nearly 80% of pooled patients in these trials. Successful reperfusion was associated with a substantial (10.4 vs. 31%) reduction in early mortality. Early thrombotic reocclusion rates varied dramatically (4 vs 31%).

In contrast to the successes of primary and secondary PTCA in the therapy of acute myocardial infarction, PTCA as adjunctive therapy to reduce the degree of residual stenosis following successful thrombolytic reperfusion has proven disappointing. The TIMI-IIA/B, TAMI-II and ECSG trials have directly addressed the role of adjunctive PTCA in the immediate, early or subacute phase of myocardial infarction following thrombolytic therapy. The results of these trials are remarkably similar; in each study, routine PTCA failed to reduce the rate of early reinfarction, recurrent ischemic events or early mortality (15-17). Routine PTCA following successful thrombolytic therapy of acute myocardial infarction, therefore, does not appear warranted.

The efficacy of PTCA in establishing reperfusion of chronically occluded coronary arteries is of limited significance to the current discussion. Success rates as high as 83% have been reported (17), although the clinical significance of this success remains unclear. If, as some data suggest, coronary reperfusion conveys important benefits separate from myocardial salvage, however, efforts to achieve reperfusion of chronically occluded epicardial coronary arteries, even in the absence of symptoms, may in some cases, be warranted.

INVESTIGATIONAL APPROACHES TO THROMBOLYSIS

The therapeutic successes of the currently available thrombolytic agents have fostered an intense interest in the molecular biology of the fibrinolytic system. Limitations of naturally occurring activators of the fibrinolytic system as therapeutic agents and an expanding knowledge of the process of fibrinolysis at a molecular level have stimulated efforts to engineer improved thrombolytic proteins. While the superiority of novel agents produced by protein engineering remains to be demonstrated in a clinical setting, studies of their molecular properties have yielded important insights into the relationship between the structure and function of components of the fibrinolytic system, and into the mechanisms that regulate the fibrinolytic process.

BIOLOGY OF THE ENDOGENOUS FIBRINOLYTIC SYSTEM

THROMBI

Thrombi are comprised in varying proportions of aggregated platelets, polymerized fibrin, "linking" proteins that cause the

platelet-fibrin skeleton to adhere to tissue surfaces, a variety of adsorbed proteins, and entrapped formed elements of blood. Formation of a stable thrombus results from three general processes: i) adherence to and activation of platelets on a thrombogenic surface; ii) activation of the coagulation cascade resulting in proteolytic cleavage of circulating fibrinogen to fibrin monomers that polymerize spontaneously; and iii) subsequent covalent cross-linking of the nascent fibrin skeleton and clot retraction.

PLATELET ADHERENCE

Platelet adherence and aggregation is mediated by several intrinsic membrane glycoproteins that link the platelet cytoskeleton to the fibrin meshwork of a thrombus and components of the vessel wall. Platelet glycoprotein (GP) II_b/III_a is a transmembrane receptor for a variety of extracellular glycoproteins containing the Arg-Gly-Asp (RGD) tripeptide motif, including fibrinogen, fibronectin, vitronectin and von Willebrand's factor (18). A related but antigenically distinct RGD receptor is found on the surface of vascular endothelial cells (19). Platelet (GP) Ib, also a transmembrane receptor, mediates binding of platelets to von Willebrand factor in the vessel wall (20). Extracellular matrix glycoproteins therefore function as bridges for platelet adherence to the vascular wall.

Additional platelets attach to the initial layer in a process termed aggregation that is dependent upon binding of fibrinogen to the GP IIb/IIIa receptor. Aggregation is accompanied by a release reaction in which a variety of substances that stimulate further platelet activation, including adenosine diphosphate, thromboxane A2, serotonin, calcium, fibrinogen and peptide growth factors, are secreted. Activated platelets also express a cell surface receptor for the prothrombinase complex, which facilitates generation of thrombin. Thrombin acts both as a potent platelet activator, and as a catalyst for proteolytic cleavage of fibrinogen (reviewed in 21).

GENERATION OF FIBRIN

Fibrinogen, an Mr 340,000 multimeric glycoprotein synthesized and secreted by hepatocytes, circulates at a concentration of 5-20 μM (1.5-4.0 mg/ml). It consists of three polypeptides, the Aα, Bβ and γ chains, assembled into a disulfide-linked hexameric structure $[A\alpha B\beta\gamma]_2$. During thrombus formation, sequential thrombin-catalyzed proteolytic cleavage of fibrinopeptides A and B (from circulating fibrinogen) produces fibrin monomers $[\alpha\beta\gamma]_2$ which rapidly polymerize. The resulting fibrin gel is loosely associated with tissue surfaces, platelets and entrapped blood constituents by noncovalent interactions and is readily subject to disruption. Stabilization of the nascent thrombus occurs by i) the factor $XIII_a$-catalyzed formation of covalent ϵ-lysyl-γ-glutamyl linkages (transglutamination) between adjacent fibrin fibers, ii) cross-

linking of fibrin to constituents of the extracellular matrix (e.g., fibronectin) and soluble plasma proteins (e.g., α_2 antiplasmin) and iii) clot retraction mediated by contractile elements of the platelet cytoskeletal apparatus.

FIBRINOLYSIS

PLASMIN(OGEN)

Fibrin is degraded enzymatically by the serine protease plasmin, which catalyzes cleavage of Lys-Xaa and Arg-Xaa peptide bonds. Plasmin is derived from the circulating, enzymatically inactive proenzyme plasminogen which is present at a concentration of 2 μM (~ 200 μg/ml). Native plasminogen, an Mr 92,000 glycoprotein synthesized by hepatocytes, possesses a glutamic acid residue at the aminoterminus (termed Glu-plasminogen (22)). Proteolytic cleavage of the internal Arg560 - Val561 peptide bond converts Glu-plasminogen into the active serine protease Glu-plasmin, a process termed plasminogen activation (23). Plasmin is a powerful protease that cleaves a wide variety of substrates in vitro. However, under physiologic conditions several mechanisms act to constrain plasmin mediated proteolysis.

Plasmin(ogen) binds to fibrin with high affinity ($K_d \sim 3x10^{-7}M$). This interaction is mediated by lysine binding sites located within the first four of five repetitive domains of plasminogen, which are termed kringles (24-26) and are organized around three internal disulfide bonds. Activation of fibrin-bound plasminogen occurs much more efficiently than activation in solution (see below). While intact fibrin has only a limited capacity to bind plasminogen, generation of small quantities of plasmin on the fibrin surface affects an amplification of the fibrinolytic cascade by two mechanisms. First, limited digestion of fibrin by plasmin exposes additional carboxyterminal lysine residues that mediate binding of additional plasminogen molecules (27). Second, plasmin catalyzes the proteolytic cleavage of an aminoterminal peptide from Glu-plasminogen to produce an Mr 83,000 form of the proenzyme with Met68, Lys77, or Val78 at the aminoterminus, collectively termed Lys-plasminogen. Lys-plasminogen binds more avidly to fibrin and is more readily activated than the native Glu-form of the proenzyme (28,29). These processes result in the preferential generation of plasmin on fibrin surfaces.

ANTIPLASMIN

The proteolytic activity of plasmin in the circulation is further constrained by the existence of a rapid plasmin inhibitor, α_2-antiplasmin (α_2-AP), an Mr 70,000 glycoprotein member of the serine protease inhibitor (serpin) gene family that circulates at a concentration of 1 μM (70 μg/ml). Plasmin and α_2-AP interact by two mechanisms. First, α_2-AP binds reversibly but with high affinity ($K_d = 10^{-9}M$, $K \sim 3 \times 10^7\ M^{-1}s^{-1}$) to the lysine binding sites present in the kringle domains of plasmin (30). Secondly, α_2-AP

functions as a suicide substrate for plasmin, undergoing proteolytic cleavage of the Arg364-Met365 peptide bond (31). Cleavage results, however, in the formation of a stable acyl-enzyme intermediate between the newly generated carboxyterminal arginine of α_2-AP and the active site serine of plasmin, resulting in the formation of an irreversibly inactivated 1:1 plasmin-antiplasmin complex. Formation of this stable inactive complex is strongly facilitated by binding of α_2-AP to the lysine binding sites of plasmin; bound α_2-AP inactivates plasmin approximately 50-fold more rapidly than free (32). It is not known whether this effect results from spatial positioning of the α_2-AP reactive center of for proteolytic attack or from other conformational effects on plasmin and/or α_2-AP.

From the kinetics of inactivation of plasmin and the concentration of α_2-AP in plasma, the half-life of plasmin in circulation is estimated at less than 0.1 second. Binding of plasmin(ogen) to fibrin, or to specific endothelial cell surface receptors (33-35) via the lysine binding sites competitively antagonizes the initial reversible binding of α_2-AP (18). Bound plasmin, therefore, is inactivated substantially less rapidly than free.

Preferential activation of bound plasmin, the potential for an amplification loop of plasminogen activation, and the efficient inactivation of free plasmin cooperatively constrain the broad proteolytic activity of plasmin in blood to the surfaces of endothelial cells or the fibrin skeleton of a thrombus (36).

ENDOGENOUS PLASMINOGEN ACTIVATORS

Two specific, endogenous activators of plasminogen have been identified.

UROKINASE

uPA is an Mr 54,000 glycoprotein initially identified in human urine. Subsequently, synthesis and secretion of UPA from a variety of primary and transformed cells has been demonstrated (for review see 37).

The secreted form of UPA is a 411 amino acid monomeric protein consisting of three structural domains. The aminoterminal portion of the molecule (residues 1-44) shows sequence homology to the epidermal growth factor precursor and similar domains, termed "EGF" domains, that occur in a variety of secreted proteins. Residues 45-133 comprise a kringle domain homologous to domains in plasminogen, prothrombin, and lipoprotein A_2. The carboxyterminal catalytic domain of UPA is homologous to other serine proteases of the chymotrypsin family, and contains the canonical catalytic triad, His204, Asp258, Ser356. The amino acid sequence of UPA inferred from the nucleotide sequence of cloned cDNAs additionally reveals a 20 residue hydrophobic signal peptide which is cleaved

from the nascent 431 amino acid polypeptide at an early stage in the secretory pathway. Following cleavage of the signal peptide, UPA is glycosylated at a single N-linked glycosylation site, Asn302, and is secreted as a single chain glycoprotein termed scuPA or prourokinase (reviewed in 38).

The domain structure of uPA reflects the exon structure of the uPA gene. The human gene encoding uPA, located on chromosome 10, spans 6.4 kb and contains 11 exons. Exon II encodes the signal peptide, exons III and IV the EGF domain, and exons V and VI the kringle domain. The catalytic domain is encoded by exons VII through IX. The alignment of intron-exon boundaries between and within the structural domains of uPA with those in genes encoding homologous domains in other blood proteins suggests that the uPA gene arose from the evolutionary assembly of exons coding for discrete structural modules in a process termed exon-shuffling (39).

Following secretion, scuPA undergoes proteolytic cleavage of the internal Lys158 -Ile159 peptide bond, converting scuPA to a two-chain, disulfide-linked form, tcuPA (40-42). While there is universal agreement that tcuPA is an active protease which efficiently (K_M = 1.4 x 10^{-6}M; K_{CAT} = 0.7 s^{-1}) activates plasminogen, the catalytic activity of scuPA has been debated (40,42-45). Since, scuPA is rapidly converted to the active two-chain form in the presence of even trace quantities of plasmin, assessment of the catalytic activity of scuPA has proven difficult. When extensive precautions have been taken to prevent plasmin-mediated conversion of scuPA to tcuPA, and in studies of recombinant scuPA mutagenized to render the protein resistant to plasmin by replacement of Lys158 (46,47), scuPA appears to activate plasminogen very inefficiently. Single-chain uPA, therefore, is physiologically a true zymogen.

As isolated from body fluids, tcuPA is heterogenous, reflecting partial proteolysis of the aminoterminal light, or A-chain. High molecular weight uPA, the Mr 54,000 form of the active serine protease, undergoes variable removal of Lys158 and Phe157. Additionally, an Mr 33,000 or low molecular weight form of uPA lacking the EGF and kringle domains is derived from the high molecular weight form by cleavage of the Lys135 - Lys136 peptide bond (48). Both HMW and LMW forms of tcuPA are catalytically active. Two-chain uPA displays little specific affinity for fibrin and efficiently activates both free and bound plasminogen (42,44). Physiologically, the plasminogen activator activity of uPA is constrained by: i) the low catalytic activity of scuPA, ii) physical localization of uPA to cell surfaces mediated by specific high-affinity cell surface receptors, and iii) the existence of specific rapid inhibitors (see below).

Several cell types including peripheral blood monocytes, fibroblasts and a variety of transformed cell lines (49-51) express a high-affinity (K_d ~ 10^{-10}M) cell-surface receptor for uPA (uPAR). Nucleotide sequencing and heterologous expression of a cloned cDNA

encoding the human fibroblast uPAR reveals a 313 amino acid protein with five potential N-linked glycosylation sites and a hydrophobic membrane spanning domain near the C-terminus (52). Peptide competition experiments implicate residues 12-32, located in the "EGF" domain of urokinase, mediate binding to the uPAR (53). Single chain uPA and tcuPA bind to the uPAR with equal affinity, and receptor-bound scuPA is readily subject to plasmin-mediated conversion to the two-chain form (54,55). Bound tcuPA is catalytically active, dissociates from the cell surface slowly ($t_{½} \geq 5$ hours), and is not subject to rapid endocytosis or degradation, thus providing a mechanism for stable, cell-associated plasminogen activator activity capable of generating a plasmin-mediated zone of pericellular proteolysis (reviewed in 37). Additionally, uPA bound to cell surface receptors of at least some cell types appears resistant to inactivation by specific inhibitors (55) although observations on other cell lines have differed (56).

Although uPA is detectable in circulation, the contribution of this enzyme to total circulating plasminogen activator activity is small. While uPA bound to the surface of cells entrapped within thrombi or residing within the vascular wall may contribute to the process of fibrinolysis under some physiologic conditions, the stable association of uPA with fibroblasts and inflammatory cells suggests that the central role of this enzyme may reside in the processes of inflammation, tissue growth and remodeling. Additionally, production and cell-surface binding of uPA by a variety of transformed cell types is consistent with an important pathophysiologic role in tumor invasion and metastasis (reviewed in 57,58).

TISSUE PLASMINOGEN ACTIVATOR

tPA is an Mr 70,000 glycoprotein synthesized and secreted by vascular endothelial cells, ovarian granulosa cells and a variety of transformed cell lines (reviewed in 58,59). As secreted, tPA shows some aminoterminal heterogeneity reflecting alternative processing in the secretory pathway (62). The amino acid sequence of TPA inferred from the nucleotide sequence of cloned CDNAS reveals a 562 amino acid polypeptide, containing an aminoterminal hydrophobic signal sequence and a short-tract of hydrophilic residues thought to comprise a "pro" sequence variably cleaved from the nascent polypeptide in the secretory pathway. Mature TPA exists predominantly as a single, 527 amino acid polypeptide chain. Like uPA, tPA consists of a series of discrete structural domains. The aminoterminal domain, spanning residues 4-50, is homologous to the "finger" domain of fibronectin. Residues of 51-87 comprise an "EGF" domain homologous to that found in uPA. Two sequential kringle domains, residues 88-175 and 176-273, precede the carboxyterminal catalytic domain, which shows homology to other members of the chymotrypsin family of serine proteases and contains the characteristic active site triad, His322, Asp371, Ser478 (reviewed in 57-61).

The human tPA gene, located on the short arm of chromosome 8, spans 29 kb. Like uPA, the boundaries between the structural domains of tPA correspond to intron-exon boundaries in the tPA gene. The single mRNA encoding tPA is 2655 nucleotides in length and contains an open reading frame of 1686 nucleotides (63-69). From the amino acid sequence, tPA contains four potential N-linked glycosylation sites, of which three, Asn117, Asn448, and variably Asn184 are glycosylated in the secreted protein (62).

As secreted, tPA is a single polypeptide chain. Proteolytic cleavage of the internal Arg275 - Ile276 peptide bond converts the single-chain to a disulfide-linked two-chain form. Unlike the single-chain forms of related serine proteases, sctPA is catalytically active (see below). Assayed in the absence of fibrin, activation of plasminogen by tPA is inefficient, reflecting a K_M (~ 65 μM) substantially above physiologic plasminogen concentration (~ 2 μM) (29). Like plasminogen, however, tPA demonstrates specific high-affinity binding to both fibrin and specific cell surface receptors (see below), and in the presence of fibrin the catalytic activity of tPA is dramatically enhanced. The binding of tPA to fibrin and the resulting enhancement of plasminogen activator activity are complex.

Direct binding studies demonstrate that both single- and two-chain tPA bind specifically (K_d ~ $4x10^{-7}$M) to a single site per fibrin monomer in intact (polymerized) fibrin (70). Binding to this site is antagonized by ϵ-aminohexyl compounds, implicating internal lysine residues in fibrin in this interaction. Partial fibrinolysis exposes additional tPA binding sites which demonstrate significantly higher affinity (K_d < 10^{-9}M). At least two classes of higher affinity sites exist; binding to one class is competitively antagonized by ϵ-amino acids and abolished by carboxypeptidase B digestion, identifying these sites as carboxyterminal lysine residues exposed by partial fibrinolysis (71).

Proteolytic and chemical modification studies have shown that the affinity of tPA for fibrin is a function of the aminoterminal heavy chain (72,73). Functional studies of variant tPA proteins engineered to delete or modify structural domains of the heavy chain have demonstrated that tPA interacts with fibrin in a complex series of events. Variant proteins lacking the finger domain demonstrate diminished binding to low concentrations of fibrin. Variants lacking both kringle domains show dramatically reduced binding, while deletion of all three of these domains produces a variant molecule with no fibrin affinity (74-79).

Kringle-mediated binding to intact fibrin is antagonized by aminohexane, implicating an aminohexyl binding site in the association of tPA with internal lysine residues in fibrin. Binding to carboxyterminal lysine high-affinity sites in partially degraded fibrin is antagonized by ϵ-aminoacids (71), and both kringle domains of tPA possess lysine binding sites (79). By contrast, binding mediated by the finger domain to either intact

(75,77) or partially degraded (71) fibrin is insensitive to ε-aminoacids; the mechanism responsible for the fibrin affinity of the finger domain is unknown.

Fibrin enhances the catalytic activity of tPA by two mechanisms. First, in the presence of fibrin, plasminogen activation occurs in a ternary complex of fibrin, plasminogen and tPA. The K_M for this reaction (~ 0.16 μM) is several orders of magnitude lower than that for activation of plasminogen in the absence of fibrin (80,81). Thus, under physiological conditions, plasminogen activation is effectively constrained to fibrin (or cell) surfaces. Secondly, fibrin alters the catalytic activity of tPA. Functional studies of recombinant tPA proteins mutagenized to destroy the cleavage site by replacement of Arg275 demonstrate that, in the absence of fibrin, the catalytic activity of two-chain tPA is greater than that of single-chain tPA due to a higher K_{CAT} for plasminogen activation (82-84). In the presence of intact fibrin (84) and some (83), but not all (82), soluble fibrin preparations, the catalytic activities of the single- and two-chain forms of the enzyme are similar, apparently reflecting a fibrin-induced change in the conformation of the single-chain form (85,86).

By analogy to the activation of chymotrypsinogen, cleavage of the Arg275-Ile276 peptide bond in tPA is thought to liberate the newly formed aminoterminus to interact with Asp477, located immediately N-terminal to Ser478 of the catalytic triad; formation of an analogous salt-bridge is apparently necessary to establish an active catalytic center in chymotrypsin (87,88). If this model of cleavage-induced activation of tPA is accurate, then the catalytic activity of sctPA likely results from an alternative mechanism to stabilize the active conformation. Studies of proteolytically modified (89) and mutagenized (88,90) tPA proteins have suggested that the ε-amino groups of Lys277 or Lys416 in the catalytic domain of tPA may interact with Asp477, substituting for an aminoterminal Ile276 in the formation of a stabilizing salt-bridge, and thereby rendering the single-chain form of the enzyme catalytically active. Variant single-chain tPA proteins in which these basic residues have been replaced by uncharged amino acids, however, retain significant catalytic activity in the presence of fibrin (90), indicating that strong ligand interactions may stabilize the catalytic center in an active conformation.

While binding to fibrin is mediated by both the finger and kringle domains of tPA, only the kringle domains participate in enhancing catalytic activity. Variant tPA proteins retaining either kringle domain demonstrate fibrin-stimulated plasminogen activator activity, while deletion of both kringle structures produces an enzyme with the catalytic properties of the isolated light chain (79). Studies of variant proteins modified by rearrangement of the heavy chain domains suggest that positioning of the kringle structures relative to the catalytic light chain is important in conveying fibrin-stimulation of catalytic activity

(91).

Thus, as with plasminogen, the functional properties of tPA suggest an important amplification of thrombolytic activity on the fibrin skeleton of a thrombus. Binding of small amounts of Glu-plasminogen and single-chain tPA to intact fibrin results in generation of small quantities of plasmin. Plasmin both cleaves polymerized fibrin exposing additional binding sites for plasminogen and sctPA, and converts circulating sctPA into the more intrinsically active two-chain form, cooperatively accelerating the generation of additional plasmin.

Because plasminogen circulates at relatively high concentration, the limiting step in initiation of the fibrinolytic cascade is the delivery of (predominantly) single-chain tPA to the fibrin skeleton of a thrombus in an active form. Several physiologic processes act to modulate the availability of active tPA: i) regulated production and release of tPA from vascular endothelial cells; ii) binding of tPA to specific cell-surface receptors; iii) clearance of tPA from the circulation; and iv) the presence in plasma of specific plasminogen activator inhibitors.

PRODUCTION AND RELEASE OF T-PA

Vascular endothelial cells are the principal source of tPA in circulation. Synthesis of tPA is regulated primarily at the level of gene transcription and several physiologic mediators exert potentially important effects on the rate of transcription of the human tPA gene (reviewed in 59 and references therein). Whether alterations in tPA synthesis play an important role in the pathophysiology of thrombotic disease, however, is uncertain.

A variety of physiologic stimuli induce release of tPA from vessel walls and/or endothelial cells in culture. The rapidity of this response implicates an intracellular (or cell-associated) pool of presynthesized tPA which is depletable by repeated stimulation. While several specific mediators capable of inducing tPA release have been identified, the molecular mechanisms involved, and the role of inducible tPA release in the maintenance of circulatory integrity have not been defined. Defective release of tPA from vascular wall has been observed on a familial basis and in a minority of patients with sporadic intravascular thrombosis (92,93).

CELL SURFACE BINDING OF T-PA

Vascular endothelial cells express a specific, saturable cell-surface receptor which binds tPA with high-affinity (K_d ~ 9 x 10^{-9}M)(77). This receptor is an Mr 40,000 intrinsic membrane protein, present at approximately 2-8 x 10^5 sites/cell, which binds tPA in an active-site independent manner. Receptor-bound tPA is catalytically active, capable of activating both free and cell-surface bound plasminogen to generate pericellular fibrinolytic

activity (34,94,95). Additionally, receptor-bound tPA appears at least relatively resistant to inactivation by specific inhibitors (see below), suggesting that a small cell-surface pool of tPA could contribute significantly to the antithrombotic activity of the intact endothelium by initiating pericellular fibrinolysis.

CLEARANCE OF T-PA

The half-life of exogenously administered tPA in the circulation is short ($t_{½}$ ~ 6 min in man). Clearance is affected principally by the liver (96,97); two specific clearance mechanisms have been implicated, one a specific hepatocyte cell-surface receptor recognizing structural components of the aminoterminal finger and "EFG" domains (98), and the second, dependent upon N-linked glycosylation, apparently recognizes mannose-rich oligosaccharide linked to Asn117. Mutant proteins lacking the aminoterminal finger and "EGF" domains and/or in which Asn117 has been replaced to destroy the N-linked glycosylation site are cleared from the circulation one to two orders of magnitude more slowly than the native protein (78,99-103). Association of tPA with specific plasma inhibitors does not appear to exert an important effect on hepatic clearance.

INHIBITORS

The specific activity of purified recombinant tPA *in vitro* is approximately 500,000 IU/mg. The concentration of tPA antigen in circulation normally ranges from 30-150 Pm (2-10 ng/ml), yet assays of normal plasma reveal circulating plasminogen activator activities ranging from .05 to .5 IU/ml, a specific activity 1-2 orders of magnitude lower than the purified enzyme *in vitro*. This discrepancy results from the existence in plasma of proteins which rapidly inactivate circulating plasminogen activators including tPA; under basal conditions only 1-10% of circulating tPA exists in a catalytically active form (104). While several plasma proteins inactivate both uPA and tPA *in vitro*, the principal physiologic inhibitor in blood is plasminogen activator inhibitor-1 (PAI-1).

PAI-1

PAI-1 is an Mr 52,000 glycoprotein synthesized and secreted by wide range of cells including vascular endothelial cells (reviewed in 105). Observations in man and several other species suggest that as much as half of circulating PAI-1 is secreted from the liver (106). The concentration of PAI-1 in circulation can vary over more than an order of magnitude, but normal circulating levels range from approximately 0.1 - 1.3 Nm.

The primary sequence of PAI-1 deduced from nucleotide sequencing of cloned CDNAS (107-109) reveals a 23 amino acid signal peptide and a mature protein of 379 amino acids with substantial homology to other members of the serine protease inhibitor (serpin) family. Potential sites for N-linked glycosylation are present at

Asn209, Asn265 and Asn329.

PAI-1 is encoded by a 9.2 kb gene containing 9 exons located on chromosome 6. Northern blotting of human endothelial cell RNA reveals mRNAs of 2.2 and 3.2 kb, reflecting alternative polyadenylation at two sites the 3'-untranslated region. While both mRNAs are expressed in cultured human cells, no important physiological correlates of the alternative mRNAs have been identified.

As with α_2-AP, PAI-1 functions as a suicide substrate for its cognate serine proteases tPA and uPA. The reactive center of PAI-1 is contained on a loop of residues apparently bound by the active site of plasminogen activators. Proteolytic cleavage of the Arg346 - Met347 peptide bond (termed P1 and P1' residues) results in the formation of a stable acyl-enzyme intermediate, producing a stably inactivated 1:1 stoichiometric protease-serpin complex. Recent functional studies of mutant recombinant tPA and PAI-1 proteins produced by oligonucleotide-directed mutagenesis have shed considerable light on the molecular interaction of tPA and PAI-1.

Unlike α_2-AP, interaction of PAI-1 with plasminogen activators is not strongly dependent upon the aminoterminal heavy-chain domains; both mutant tPA molecules lacking the heavy-chain and the low molecular weight form of uPA demonstrate rates of inactivation by PAI-1 which are similar to the native proteins (79,100-102). PAI-1-mediated inactivation is dependent upon catalytic activity, as mutant plasminogen activators rendered catalytically inactive by modification (34) or replacement (113) of the active-site serine residue do not form stable complexes with PAI-1. Similarly, destruction of the reactive center of PAI-1 by replacement of Arg346 by residues other than lysine, dramatically reduces inhibitor activity (RD Gerard, in press).

Molecular modeling based upon the crystal structure of the trypsin-bovine pancreatic trypsin inhibitor complex and alignment of the primary amino acid sequences of trypsin and the catalytic domain of tPA have been employed to identify amino acid residues in tPA and PAI-1 that mediate the molecular interaction of these proteins. A loop of residues, positions 296-304 in the tPA molecule, predicted to reside near the surface of the active site, contains four basic amino acids K296, R298, R299, and R304. The reactive center loop of PAI-1 contains the acidic residues E350 and E351 in the P4' and P5' positions. Deletion of the 296-302 loop of the tPA molecule, or to a lesser extent, replacement of the individual basic residues R298, R299, or R304 by glutamic acid residues renders the resulting mutant tPA molecule resistant to PAI-1 inactivation (114,115). Similarly, replacement of the P4' and P5' glutamic acid residues of PAI-1 by basic arginine residues restores plasminogen activator inhibitor activity toward a serpin-resistant tPA (R304→E) (RDG et al, in press). These observations suggest that complementary charged residues bordering the active site of tPA and the reactive center of PAI-1 facilitate the

interaction of these proteins, positioning the reactive P1 Arg-P1' Met peptide bond for proteolytic attack by the active site serine residue. By analogy to the crystal structure of the cleaved form of a related serpin, α1-antitrypsin, cleavage of the P1-P1' peptide bond in PAI-1 is predicted to result in a dramatic conformational change in the molecule, presumably rendering the acyl-enzyme intermediate resistant to hydrolysis and thus producing a covalently associated complex.

In vitro, PAI-1 rapidly inactivates both uPA ($K \sim 10^8 M^{-1}s^{-1}$) and tPA ($K \sim 10^7 M^{-1}s^{-1}$) (110-112). From the kinetics of inactivation of tPA and the concentrations of tPA and PAI-1 in plasma, the estimated half-life of active tPA in circulation is approximately 100 sec; within minutes of secretion the majority of tPA in plasma is therefore converted to the inactive tPA-PAI-1 complex, explaining the lower specific activity of the circulating pool of tPA in comparison to the purified recombinant enzyme. For several reasons, however, circulating plasminogen activator pool may not accurately reflect the availability of active plasminogen activator under physiologic/pathophysiologic conditions:

i) The interaction of tPA and PAI-1 while rapid, is substantially slower than the inactivation of plasmin by α_2-antiplasmin. Therefore, stimuli which induce release of tPA from vascular endothelium, while raising plasma tPA antigen levels to a minor extent, can transiently produce substantial increases in plasminogen activator activity since the specific activity of the newly released tPA is several hundred-fold greater than that of the circulating pool (116,117).

ii) Some observations suggest that tPA bound to fibrin, or to cell-surface receptor may be relatively resistant to serpin inactivation, and thus the specific activity of the circulating plasminogen activator pool may not accurately reflect the available endogenous plasminogen activator potential.

iii) The size of the pool of PAI-1 available to inactivate plasminogen activators is similarly difficult to estimate for several reasons. First, PAI-1 is an acute phase reactant, with plasma concentrations increasing several-fold in pregnancy or in response to infection, surgical procedures or a variety of systemic illnesses (118). Secondly, PAI-1 binds to components of extracellular matrix, and matrix-associated PAI-1 retains plasminogen activator inhibitor activity (119). Currently, no accurate estimate of the size of the bound, extracellular PAI-1 pool is available. Thirdly, PAI-1 is stored in the α-granules of platelets and released by platelet activation (120.121), and the pool of platelet PAI-1 is several-fold larger than that in free circulation. Finally, PAI-1 exists in both active and inactive or latent forms. PAI-1 is apparently secreted as an active serpin. *In vitro*, inhibitor-specific activity declines spontaneously at 37°C. Denaturation of "latent" PAI-1 by heat to potent chaotropic agents (122) or exposure phospholipid vesicles (123) can reactivate the serpin, and high concentrations of arginine can

stabilize the active form of PAI-1. The extent to which latency and reactivation are important *in vivo* is uncertain, although apparent reactivation of exogenously administered "latent" PAI-1 has been observed (124).

Consequently, accurate estimation of systemic, or local fibrinolytic potential under physiologic conditions is difficult. Nonetheless, several observations suggest that PAI-1 is an important physiologic modulator of plasminogen activator activity. Elevated PAI-1 levels have been observed in patients with familial thrombotic syndromes, and have been associated with risk for venous thrombosis, acute (125) or recurrent (116) myocardial infarction, and early thrombotic graft occlusion following coronary bypass surgery (126).

OTHER PLASMINOGEN ACTIVATOR INHIBITORS

Under normal physiologic conditions, PAI-1 accounts for nearly all the plasminogen activator inhibitor activity in plasma. Several other proteins, however, can inactivate plasminogen activators *in vitro*, or after therapeutic administration. PAI-2 is an Mr 48,000 glycoprotein initially identified in the serum of pregnant women. While a rapid inhibitor of uPA ($K \sim 10^7 M^{-1}s^{-1}$), PAI-2 inhibits tPA slowly ($K \sim 10^5 M^{-1}s^{-1}$). Moreover, even during pregnancy, PAI-2 accounts for a small fraction of the plasminogen activator inhibitor activity in human plasma. Both α_2-antiplasmin and α_2-macroglobulin form stable inactive complexes with tPA at pharmacologic concentrations. While these inhibitors can contribute to inactivation of therapeutically administered plasminogen activators, the kinetics of inactivation are slow ($K < 10^4 M^{-1}s^{-1}$) (reviewed in 105).

THERAPEUTIC THROMBOLYSIS

EXOGENOUS PLASMINOGEN ACTIVATORS: STREPTOKINASE

Streptokinase is an Mr 47,000 protein produced by Lancefield group C strains of β-hemolytic streptococci. Streptokinase possesses no intrinsic enzymatic activity. Rather, it binds to plasminogen specifically, rapidly ($K \sim 3 \times 10^7 M^{-1}s^{-1}$) and with high affinity ($K_d \sim 5 \times 10^{-11} M$). Binding of streptokinase to plasminogen renders the zymogen catalytically active, and the streptokinase-plasminogen complex is autocatalytically converted to streptokinase-plasmin. This complex then catalyzes the conversion of circulating plasminogen to plasmin (127,128).

The streptokinase-plasminogen complex shows fibrin affinity, and fibrin-bound plasminogen complexes with streptokinase more readily than does the free proenzyme (129). The K_M for plasminogen activation in solution ($K_M = 0.12\ \mu M$) however, is substantially less than the circulating plasminogen concentration. Additionally, the half-life of the streptokinase-plasminogen complex is short, owing to rapid autocatalytic proteolysis of the streptokinase

moiety (128) which destroys the plasminogen activator before significant binding to polymerized fibrin can occur. Thus, *in vivo*, streptokinase produces an effectively nonselective plasminogen activator activity, and streptokinase-induced thrombolysis is therefore dependent upon the generation of a systemic fibrinolytic state.

LIMITATIONS OF THE NATURALLY OCCURRING PLASMINOGEN ACTIVATORS AS THERAPEUTIC AGENTS

As discussed previously, the naturally occurring plasminogen activators have become important therapeutic agents in the treatment of acute myocardial infarction. If administered shortly after the onset of symptoms, thrombolytic therapy successfully reestablishes coronary patency in approximately 70% of patients (130-134). Early coronary reperfusion can limit the extent of myocardial injury, improve residual ventricular function, and reduce mortality (135-140). These agents are not, however, uniformly effective, failing to produce coronary reperfusion in approximately 25% of patients with acute myocardial infarction. An additional 15-25% of arteries successfully reperfused suffer early thrombotic reocclusion. Time to reperfusion is the most important determinant of the extent of myocardial salvage. Even with aggressive administration protocols, coronary thrombolysis with available agents requires 45-90 min; more rapidly effective agents, if available, would have obvious attraction. Additionally, each of the naturally occurring plasminogen activators are subject to specific limitations as therapeutic agents.

STREPTOKINASE

Human plasma contains antibodies reactive against streptokinase, presumably reflecting prior streptococcal infection. Allergic reactions occur in 1.5-18% of treated patients, and are on occasion, severe. Recent streptococcal infection or prior therapeutic exposure to streptokinase can induce high titers of neutralizing antibody, reducing therapeutic efficacy.

Administered streptokinase complexes rapidly with circulating plasminogen, producing a systemic activation of the fibrinolytic system which degrades circulating fibrinogen. Polymerized fibrin in a thrombus is degraded more slowly. Because streptokinase is rapidly degraded, prolonged infusion is required to sustain fibrinolytic activity, and circulating fibrinogen is generally depleted to less than 10% of pretreatment levels. The resulting hemorrhagic tendency persists until hepatic synthesis restores the circulating fibrinogen pool. While not clearly associated with a greater risk of acute hemorrhagic complications (139), the prolonged period of fibrinogen depletion can complicate subsequent invasive procedures.

U-PA

By contrast to streptokinase, uPA is non-antigenic and

allergic reactions and antibody-mediated resistance are not encountered. Rapid inactivation of uPA by PAI-1, however, requires administration of quantities exceeding saturation of the endogenous inhibitor. uPA is also cleared from the circulation rapidly, requiring a sustained infusion to achieve therapeutic thrombolysis. Finally, like streptokinase, uPA produces a systemic fibrinogenolytic state, and thus a prolonged hemorrhagic tendency.

Single-chain uPA, while having low catalytic activity, possesses high affinity for plasminogen. scuPA catalyzes production of small quantities of plasmin that cleave the single-chain form to the efficient plasminogen activator tcuPA *in vivo*. By contrast to tcuPA, the plasminogen activator activity of scuPA is therefore somewhat fibrin-selective, perhaps reflecting either displacement of a bound plasma inhibitor by fibrin or affinity for fibrin-bound plasminogen (see above). The (relatively) minor degree of fibrin selectivity thus resulting has not produced clearly important therapeutic advantage (141).

T-PA

Because of the fibrin/cell surface selectivity of tPA-mediated plasminogen activation, effective thrombolytic doses produce less fibrinogenolysis than streptokinase or uPA. While in clinical trials this is not translated into a clearly lower incidence of hemorrhagic complications, the hemorrhagic tendency is temporally limited and more easily reversed. Effective thrombolysis with tPA is dependent upon achieving levels of free tPA in circulation, requiring doses in excess of inhibitor saturation (142,143). Moreover, tPA is cleared rapidly, and prolonged infusion is necessary to sustain therapeutic levels.

SECOND GENERATION THROMBOLYTIC AGENTS

Efforts to develop more efficacious thrombolytic agents have therefore targeted increases in catalytic activity, fibrin specificity and/or biological half-life. A variety of approaches have been employed to produce "second-generation" plasminogen activators, a large number of which have now been characterized, at least *in vitro*.

ACYL-PLASMINOGEN STREPTOKINASE ACTIVATOR COMPLEX

Although the complex of streptokinase and plasminogen demonstrates affinity for fibrin comparable to that of tPA, the catalytic activity of free streptokinase-plasminogen complex and autocatalytic degradation of the streptokinase moiety preclude a fibrin selective thrombolytic effect. Acylation of the active site serine of plasminogen, however, blocks both fibrinolytic activity and autocatalytic degradation of the complex (128,129). "Proactivator" complexes prepared *in vitro* by acylative trapping of the nascent streptokinase-plasminogen complex persist *in vivo* for periods sufficient to permit significant binding to polymerized fibrin. The acyl-plasminogen-streptokinase complex then undergoes

spontaneous deacylation with a half-life dependent upon the acyl group, resulting in sustained *in vivo* production of a fibrin-selective plasminogen activator activity. Anisoyl-plasminogen-streptokinase (APSAC, anistreplase) deacylates with a $t_{½}$ of 40 minutes (129), producing sustained, effective thrombolytic activity after bolus injection with less fibrinogenolytic activity than comparable doses of streptokinase (130). Some data suggest that the incidence of early thrombotic reocclusion after coronary thrombolysis with anistreplase is low, perhaps reflecting persistent low-level generation of active plasminogen activator (130). Antibody-mediated resistance and allergic reactions remain potential, if infrequent, limitations.

ENGINEERED VARIANTS OF PLASMINOGEN ACTIVATORS

The initial variants of tissue plasminogen activator were produced by deletion of structural domains in the heavy chain using naturally occurring restriction sites (71). More recent deletion and substitution mutants have been produced by site-specific, oligonucleotide-directed mutagenesis. Characterization of a large number of variant tPA molecules generated by heterologous expression of the manipulated cDNAs have identified several variant molecules with properties that could potentially improve thrombolytic efficacy.

Deletion of the EGF (102) or finger and EGF (78, 101-103) domains of tPA decrease the rate of hepatic clearance, prolonging the $t_{½}$ in circulation by three to ten-fold in experimental animals following bolus injection. Consistent with an important role for the finger domain in fibrin binding, however, variants lacking this domain demonstrate diminished fibrin affinity (74, 77-79), and reduced fibrinolytic potency at limiting activator concentrations *in vitro* (77). By contrast, introduction of a novel N-linked glycosylation site into the EGF domain of tPA diminishes hepatocyte uptake *in vitro* and prolongs clearance in experimental animals without diminishing thrombolytic activity in clot-lysis assays (144). Assays of in *vivo* thrombolytic activity have not yet been reported.

In some but not all animal species, deglycosylated variants of tPA, produced either by expression in the presence of tunicamycin (143) or by site-directed mutagenesis to eliminate the N-linked glycosylation sites Asn117, Asn184 and Asn448 (90,100), demonstrate prolonged clearance. The effect of deglycosylation on clearance apparently results from loss of the mannose-rich oligosaccharide at Asn117 (99,142), as selective enzymatic deglycosylation of this site reduces the rate of clearance in experimental animals (99).

TPA devoid of N-linked oligosaccharide demonstrates higher fibrin affinity and fibrinolytic activity in clot lysis assays than the glycosylated native protein (143). Similarly, nonglycosylated mutant proteins lacking the finger and "EGF" domains (ΔFE1X, ΔFE3X, (100,103) also demonstrate improved fibrin affinity, suggesting that deglycosylation partially compensates for the absence of the

fibrin-binding finger domain. Moreover, these proteins demonstrate higher fibrinolytic versus fibrinogenolytic activity than either native tPA or the glycosylated form of the FE deletion protein. In animal models of coronary thrombosis, ΔFE3X and ΔFE1X demonstrate higher specific thrombolytic activity than native tPA, and affect experimental coronary thrombolysis after bolus injections at doses which produce less systemic fibrinogen depletion (103).

Achievement of coronary thrombolysis with tPA is dependent upon delivery of tPA to the fibrin skeleton of a thrombus in an active form. Early thrombotic reocclusion following successful thrombolysis correlates with the disappearance of enzymatically active tPA from the circulation (142). As mentioned previously, elevated plasma PAI-1 concentrations have been correlated with the risk of coronary thrombosis in a variety of settings (above). Circulating PAI-1 levels increase as an acute phase reactant in the setting of myocardial infarction (104), and might limit therapeutic efficacy or promote early thrombotic reocclusion following therapeutic administration of tPA (142).

A tPA molecule lacking a loop of residues, positions 296-302, predicted by molecular modeling to border the active site, shows substantial resistance to inactivation by PAI-1 *in vitro* ($K \sim 3 \times 10^3 M^{-1}s^{-1}$ versus $K \sim 1 \times 10^6 M^{-1}s^{-1}$ for native rtPA,(114,115)). A mutant protein in which all three positively charged residing in this loop are replaced by negatively charged residues (K296, R298, R299 → EEE) shows even greater resistance to serpin inactivation ($K \sim 5 \times 10^2 M^{-1}s^{-1}$) presumably reflecting repulsive charged-pair interactions with glutamate residues residing near the reactive center of PAI-1 (115). These recombinant mutagenized tPA proteins demonstrate a specific activity two-three orders of magnitude greater than native tPA in human plasma, suggesting the potential for greater thrombolytic efficacy/potency *in vivo*.

From the current understanding of structure-function relationships of tPA, therefore, it is possible to produce tPA molecules with prolonged clearance, greater fibrin selectivity, and/or resistance to serpin inactivation than the native protein. The clinical efficacy of these mutagenized, recombinant plasminogen activators remains to be investigated.

A variety of approaches have been utilized in efforts to engineer uPA to improve fibrinolytic activity and affect affinity for fibrin. Initial efforts employed chemical cross-linking techniques to generate hybrid plasminogen activators, joining the aminoterminal fibrin-binding heavy chain of plasmin to the catalytic chains of uPA or tPA (145-147). While plasmin-uPA and -tPA hybrid plasminogen activators show greater fibrin affinity than native uPA, and in some cases modest increases in catalytic activity in the presence of fibrin, plasminogen activator activity is diminished.

More extensive series of chimeric recombinant plasminogen

activators have been generated by manipulating cloned cDNAs to link sequences encoding all or parts of the heavy chains of tPA and/or uPA and the catalytic domain of uPA. In this manner, chimeras with various hybrid heavy chain domain arrangements have been produced by heterologous expression, and characterized in amidolytic and clot lysis assays (148-152). Chimeric plasminogen activators have, in general, showed plasminogen activator activity comparable to uPA, and those containing the kringle domains of tPA have shown affinity for fibrin. However, stimulation of plasminogen activator activity in the presence of fibrin, is absent or minor in comparison to native tPA, implicating fibrin-induced interaction between the heavy and light chains of the native protein.

Efforts to convey fibrin affinity upon uPA using antifibrin antibodies have produced more promising results. A hybrid plasminogen activator formed by cross-linking uPA to a monoclonal antibody specific for the aminoterminal region of the β-chain of fibrin shows both fibrin affinity and significantly increased *in vitro* clot-lysis activity in comparison to the native enzyme (153). Similar results were obtained with a tPA-monoclonal antibody hybrid, which additionally showed more potent and fibrin-specific thrombolytic activity *in vivo* (154,155).

An antibody-targeted chimeric plasminogen activator has also been produced from a recombinant gene linking cDNA sequences coding for tPA to the rearranged heavy-chain gene encoding the anti-fibrin monoclonal antibody 59D3. Expression of this construct in a myeloma cell line expressing only the fibrin-specific light chain results in secretion of an antibody-plasminogen activator fusion protein. The recombinant protein demonstrates enhanced fibrin affinity and relatively preserved catalytic activity *in vitro* (156).

Recently, an antibody-plasminogen activator hybrid protein formed by cross-linking single-chain uPA and $[F_{AB}]_2$ fragment of the antifibrin β-chain monoclonal antibody has been demonstrated to possess greater *in vitro* fibrinolytic potency than native single-chain uPA, tcuPA or native tPA. Moreover, in a rabbit jugular vein model of intravascular thrombosis, the hybrid plasminogen activator was nearly 30-fold more potent than parental scuPA, while producing less fibrinogenolysis (157).

ADJUNCTIVE AGENTS

Despite aggressive or sustained infusion protocols, early intervention to reduce residual coronary stenosis, and the routine use of aspirin and systemic anticoagulation with heparin as adjuncts to thrombolytic agents in the treatment of myocardial infarction, the rates of both primary failure and early thrombotic reocclusion have remained approximately 20-30%. An expanded understanding of the process of thrombus formation at a molecular level has produced several novel strategies which show promise in experimental models of arterial thrombosis and/or thrombolysis.

Platelet surface glycoprotein IIb/IIIa, by mediating the interaction of platelets with extracellular matrix glycoproteins and protein constituents of a thrombus, plays a central role in the formation of a stable thrombus. Additionally, activation of platelets by ADP, epinephrine, collagen and thrombin are dependent upon binding of fibrinogen to glycoprotein IIb/IIIa (158). A monoclonal antibody, 7E3, which binds to glycoprotein IIb/IIIa, antagonizes binding of proteins containing the RGD tripeptide motif. This monoclonal antibody, and $F(ab')_2$ fragments derived therefrom, are potent inhibitors of platelet aggregation *in vitro* and antagonize arterial thrombus formation in experimental animals (159). When administered with rtPA to animals with experimental coronary thrombosis, 7E3 $F(ab')_2$ improves thrombolytic efficacy, reduces time to reperfusion, and antagonizes early thrombotic reocclusion (160-162). Similarly, the tetrapeptide RGDY which competitively antagonizes binding of fibrinogen to glycoprotein IIb/IIIa also prevents thrombotic reocclusion following rtPA in experimental coronary thrombosis (163).

Thrombin, in addition to catalyzing cleavage of fibrinogen to fibrin monomers, functions as an agonist of platelet activation. While heparin, a cofactor for antithrombin III mediated inhibition of thrombin, effectively antagonizes the catalytic activity of thrombin in high concentrations, usual therapeutic concentrations produce only partial inhibition, and may not effectively antagonize thrombin-mediated platelet activation. As after clinical thrombolysis, heparin is only partially effective in antagonizing platelet-dependent arterial thrombosis in experimental models. Specific, direct-acting inhibitors of thrombin, including the competitive inhibitor argatroban (164) and recombinant desulphatohirudin (165), a peptide which binds and irreversibly inactivates thrombin, strongly antagonize arterial thrombus formation in several experimental models. Whether such potent antithrombotic agents can improve the efficacy of thrombolytic therapy without unacceptable hemorrhagic risk is undetermined.

GENE-BASED THROMBOLYTIC THERAPY

Recently, gene transfer techniques have been employed to genetically modify vascular endothelial cells to overexpress plasminogen activator activity. Endothelial cells infected with recombinant retroviruses containing an engineered tPA gene have been demonstrated to stably express tPA at levels up to two orders of magnitude greater than uninfected cells in culture (166). Endothelial cells modified by retroviral-(167), and adenoviral-(unpublished observations) mediated gene transfer have been successfully reimplanted by growth on vascular grafts (167) or by seeding of denuded vascular segments (168), and stable expression of transferred reporter genes *in vivo* demonstrated. It seems likely that endothelial cells modified by gene transfer techniques to overexpress plasminogen activator activity can be successfully introduced into selected vascular segments. Direct introduction of a recombinant gene into vascular endothelium, and subsequent

expression of the transferred gene in the vascular wall has similarly been observed (169, Willerson et al, personal communication). The underlying hypothesis that local overexpression of plasminogen activator activity might exert a protective effect against intravascular thrombosis is currently unproven. Overexpression of a variety of antithrombotic products in the vascular wall might ultimately result in the stable alteration of the physiologic substrate for intravascular thrombus formation, and thus be protective against de-novo or recurrent coronary thrombosis.

THROMBOLYTIC THERAPY OF MYOCARDIAL INFARCTION: CURRENT DOCTRINE

While the salutary effect of thrombolytic therapy on mortality following myocardial infarction is now unquestioned, the reason(s) for this effect are less clearly established. In view of the important effect of residual post-infarction left ventricular function on subsequent prognosis and the potential of coronary reperfusion to achieve significant myocardial salvage, it has been widely assumed that limitation of infarct size is the mechanism by which improved survival is realized. Quoting one recent prominent review (3):

> *The mechanism leading to improved survival after thrombolytic therapy is well understood and is supported by animal experiments and data from studies in patients:*
>
> 1. *Most infarcts are caused by sudden occlusion of a major coronary artery.*
> 2. *Occlusion leads to ischemia and to cell death in a few hours.*
> 3. *Reperfusion can be achieved by intravenous and/or intracoronary administration of a thrombolytic drug, and/or by mechanical perforation and percutaneous transluminal coronary angioplasty (PTCA).*
> 4. *Timely reperfusion prevents cell death and preserves myocardial function.*
> 5. *Limitation of infarct size and preservation of regional and global ventricular function reduces early mortality and improves prognosis.*
> 6. *The beneficial effects of thrombolytic therapy will diminish with time. When all cells in the infarcted zone are irreversibly damaged, reperfusion will not result in preservation of function and improved survival.*

Predicated on the assumption that myocardial salvage is responsible for the salutary effects of coronary reperfusion on survival, current doctrine recommends thrombolytic therapy for:

> *Patients less than 70 years of age who present with chest pain consistent with the diagnosis of acute myocardial infarction and at least 0.1 mV of ST segment elevation in at least two*

contiguous ECG leads in whom treatment can be initiated within 6 hours of pain and who lack contraindications to thrombolytic therapy (7).

Several observations, however, raise questions concerning the window of opportunity for myocardial salvage. Data from the large multicenter trials of thrombolytic therapy suggest that patients receiving thrombolytic agents as late as 24 hours following the onset of symptoms have improved survival (Figure 7). The magnitude of the effect of thrombolytic agents on survival is large relative to the degree of preservation of ventricular function (Figures 2,3 and references therein).

Finally, recent observations suggest that patency of the infarct-related artery may exert important effects on post-infarction survival independent of global left ventricular function. Drs. Lange, Hillis and colleagues in the Cardiac Catheterization Laboratory at this institution have examined survival in a retrospective series of patients undergoing catheterization after suffering acute myocardial infarction (169-171). In patients with both single-, and multivessel coronary artery disease, patency of the infarct related artery at the time of catheterization is a strong predictor of long-term survival in patient populations with similar left ventricular ejection fractions. These studies further demonstrate that the incidence of late depolarizations on a signal-averaged ECG, predictive in post-infarct patients of inducibility of ventricular dysrhythmias during invasive electrophysiologic testing, were observed 40% of patients with an occluded infarct-related artery, and only 8% of patients in whom the infarct-related artery was patent at the time of catheterization. Notably, most of the 4-10% annual mortality observed in patients surviving the acute phase of myocardial infarction reflects sudden cardiac death (7).

CONCLUSIONS

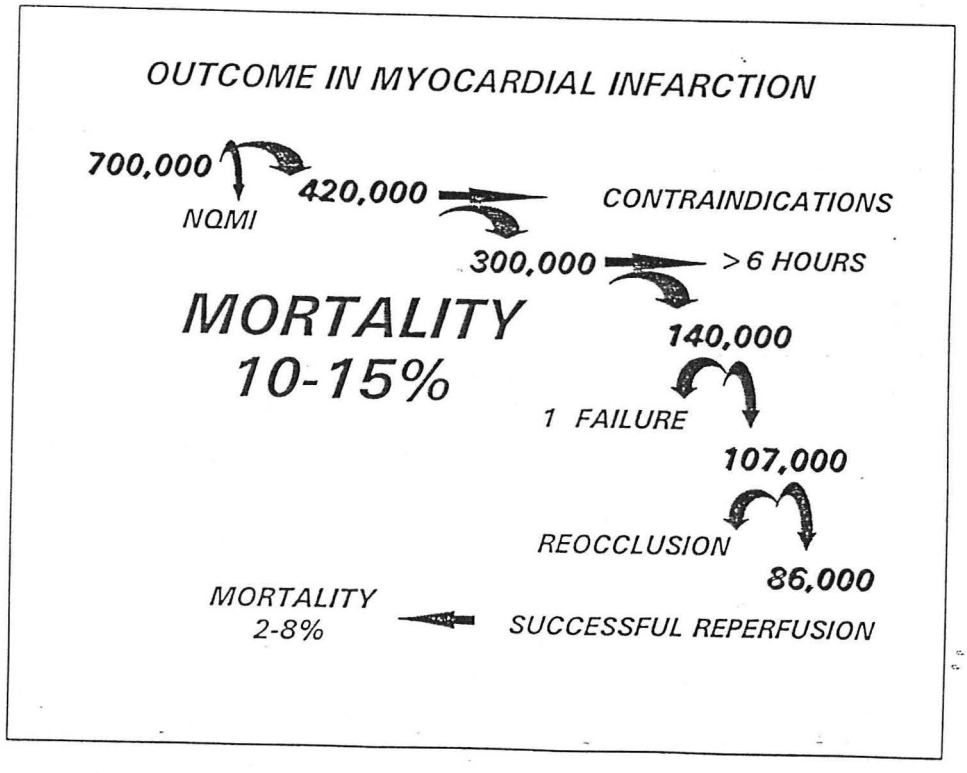
The development of successful strategies to achieve coronary reperfusion has had a profound effect on the management and prognosis of patients suffering acute myocardial infarction. Currently, however, thrombolytic therapy and/or emergent primary PTCA are largely reserved for patients presenting within 6 hours of the onset of symptoms, a period corresponding to the percieved window of opportunity for myocardial salvage.

Approximately 700,000 patients suffering acute myocardial infarction present to U.S. hospitals annually. Figure 8 illustrates schematically the outcome for these patients. By current protocol, roughly 20% of these patients are candidates for thrombolytic therapy, and in approximately 12% successful coronary reperfusion would be expected if all eligible patients were treated. Some clinical data suggest that our current approach to coronary reperfusion, based on the concept of myocardial salvage, may be too restrictive, and that important survival benefits might derive from efforts to achieve reperfusion in a much larger

EFFECT OF TIME TO TREATMENT ON MORTALITY

	% MORTALITY	
0-6 HOURS	CONTROL	TREATED
GISSI	12.8%	10.2%
ISIS-2	12.3%	8.2%
POOLED	20%	17%
6-12 HOURS		
GISSI	13.9%	13.5%
ISIS-2	12.1%	10.4%
POOLED	21%	18%
12-24 HOURS		
ISIS-2	10.8%	8.7%
POOLED	22%	12%

Figure 7



fraction of this population.

Despite aggressive treatment protocols and the addition of a variety of adjunctive therapies, the rates of primary and secondary failure to achieve stable coronary reperfusion have plateaued. Molecular engineering and an improving understanding of the processes of thrombosis and thrombolysis at a molecular level, however, are producing novel approaches with promise to improve upon current results. The challenge is to remove the event horizon of myocardial infarction.

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