New Approaches to Thrombolytic Therapy

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Tissue-type plasminogen activator (t-PA), purified from the culture fluid of a stable human melanoma cell line, is a serine protease, different from urokinase, with a molecular weight of about 70,000. It is composed of one polypeptide chain, which is converted to a two-chain molecule by limited plasmin action.

Activation of plasminogen to plasmin occurs by cleavage of the Arg 560-Val 561 peptide bond. Kinetic analysis has shown that the activation obeys Michaelis-Menten kinetics and that the presence of fibrin strikingly enhances the activation rate by increasing the affinity of plasminogen for fibrin-bound t-PA. The directed action of plasmin toward fibrin in vivo, might be explained by the low Michaelis constant in the presence of fibrin (0.16 μM), which allows efficient plasminogen activation on a fibrin clot, while its high value in the absence of fibrin (65 μM) prevents efficient activation in plasma. Plasmin formed on the fibrin surface would then be protected from rapid inactivation by α2-antiplasmin.

An important consequence of this molecular model for physiological fibrinolysis is that specific thrombolysis is only expected with the use of a specific plasminogen activator, which confines activation to the fibrin surface. Studies on the thrombolytic properties of purified t-PA in various animal species and in humans have revealed a higher specific thrombolytic activity than urokinase. Thrombolysis could be achieved without causing significant plasminogen activation, α2-antiplasmin consumption, or fibrinogen breakdown. Alternatively, pro-urokinase, the zymogen precursor of urokinase, also displays a certain degree of fibrin specificity. Its mechanism of action and potential therapeutic value remain to be established.

The main role of the fibrinolytic enzyme system in the blood is the removal of fibrin from the vascular bed. The system comprises a proenzyme, plasminogen, which can be activated to the active enzyme plasmin, which will degrade fibrin by several different types of plasminogen activators. All plasminogen activators studied so far exert their action through hydrolysis of the Arg 560-Val 561 peptide bond in plasminogen. Inhibition of the fibrinolytic system may occur at the level of plasmin or at the level of the activators.

During the last few years, significant progress has been made in our understanding of the molecular mechanisms involved in the regulation of the fibrinolytic enzyme system in vivo. This article will mainly deal with our present knowledge about the tissue type plasminogen activator (t-PA) and pro-urokinase (pro-UK) and their importance for physiological and therapeutic thrombolysis.

Physicochemical Properties of Tissue-Type Plasminogen Activator

The first satisfactory purification of t-PA from human tissues was obtained from the human uterus. With use of an antiserum raised with this preparation, it has been shown that the tissue-type plasminogen activator, the vascular plasminogen activator, and the blood plasminogen activator are immunologically identical, but different from urokinase. It is therefore assumed that the plasminogen activator found in the blood represents the vascular plasminogen activator that is released upon certain stimuli or trauma. The mean level of t-PA antigen in human plasma at rest is 6.6 ± 2.9 ng/ml, of which only about one-third represents active t-PA. Researchers have purified t-PA from the culture fluid of a human melanoma cell line in sufficient amounts to study its biochemical and biological properties. Recently, the gene of human t-PA was cloned and expressed. Human t-PA, obtained by an expression of recombinant DNA in eukaryotic cells, was shown to be indistinguishable from the natural activator isolated from human melanoma cell cultures with respect to biochemical properties, turnover in vivo, and specific thrombolytic effect.

It has been shown that t-PA is a serine protease with a molecular weight of about 70,000, composed
of one polypeptide chain containing 527 amino acids, 35 cysteine residues, and three potential N-glycosylation sites (Asn 118, 186, and 448). Upon limited plasminic action, the molecule is converted to a two-chain activator linked by one disulfide bond.\(^7\)\(^1\) This occurs by cleavage of the Arg 275-ile-276 peptide bond yielding a heavy chain derived from the NH\(_2\)-terminal part of the molecule and a light chain comprising the COOH-terminal region. The heavy chain contains two regions of 82 amino acids each (residues 92-173 and 180-261) that share a high degree of homology with the five kringles of plasminogen (triple-loop disulfide structures) and with similar kringles in prothrombin and urokinase. The catalytic site located in the light chain of t-PA is formed by the His 322, Asp 371, and Ser 478 residues. The amino acid sequences surrounding these residues are highly homologous to corresponding parts of other serine proteases such as trypsin, thrombin, plasmin, and elastase.\(^9\)\(^1\)

Comparison of the primary structures of high molecular weight urokinase and t-PA has revealed a high degree of homology between the two proteins, except that t-PA contains a 43-residue long amino-terminal region which has no counterpart in urokinase; this segment was shown to be homologous with the finger-domains responsible for the fibrin-affinity of fibronectin. Limited proteolysis of this region leads to a loss of the fibrin-affinity of the enzyme.\(^1\)\(^3\) The sequence representing residues 44-91 of t-PA was also found to be homologous with high molecular weight urokinase (residues 5-49), bovine factor X (residues 54-88), bovine factor IX (residues 54-87), bovine protein C (residues 52-96), murine epidermal growth factor (residues 2-49), and human epidermal growth factor (residues 2-49). Mainly cysteine residues are highly conserved in all these proteins.\(^1\)\(^3\)

The one-chain and two-chain forms of t-PA have different amidolytic activities toward low molecular weight substrates.\(^1\)\(^4\) They have, however, virtually the same fibrinolytic activity (clot lysis) in a purified system; the plasminogen-activating properties are also similar.\(^1\)\(^1\)\(^5\) It has been observed that on the fibrin surface, the one-chain activator is quickly converted to a two-chain form and therefore it has been suggested\(^1\)\(^5\) that physiological fibrinolysis induced by native one-chain t-PA nevertheless occurs mainly via a two-chain derivative.

Evidence was recently obtained\(^1\)\(^6\)-\(^1\)\(^9\) for the occurrence of four different types of heterogeneity of t-PA in human melanoma cell cultures. First, there is the one-chain form that can be cleaved proteolytically into a two-chain form, as just discussed. Second, both the one-chain and the two-chain molecules exhibit two forms with molecular weight differences of about 3,000 but with similar enzymatic activity.\(^1\)\(^7\)\(^1\)\(^8\) It has been shown\(^1\)\(^8\) that the 63,000 dalton molecule (type I) is glycosylated at positions 118, 186, and 448, while the 60,000 dalton form (type II) is glycosylated only at positions 118 and 448. Third, the single-chain activator shows NH\(_2\)-terminal heterogeneity; about 50% of the molecules start with an NH\(_2\)-terminal sequence Gly-Ala-Arg-Ser-Tyr-Gln- (termed L-chains), while the tripeptide Gly-Ala-Arg is absent in the other structure (termed S-chains, starting with Gly/Ser-Tyr-Gln). Finally, results compatible with a positional microheterogeneity (Gly/Ser exchange) were obtained\(^1\)\(^9\) at a single position (position L-4 which is equal to S-1).

### Mechanism of Action of Tissue-Type Plasminogen Activator

It has been shown\(^2\)\(^0\)-\(^2\)\(^1\) that t-PA is a poor enzyme in the absence of fibrin, but that the presence of fibrin strikingly enhances the activation rate of plasminogen. This has been explained\(^2\)\(^2\) by an increased affinity of fibrin-bound t-PA for plasminogen (Michaelis constant 65 \(\mu\)M in the absence, and 0.16 \(\mu\)M in the presence of fibrin) without significantly influencing the catalytic efficiency of the enzyme. The kinetic data of Hoylaerts et al.\(^2\)\(^6\) support a mechanism in which fibrin provides a surface to which t-PA and plasminogen adsorb in a sequential and ordered way yielding a ternary complex. Fibrin essentially increases the local plasminogen concentration by creating an additional interaction between t-PA and its substrate. The high affinity of t-PA for plasminogen in the presence of fibrin thus allows efficient activation on the fibrin clot, while no efficient plasminogen activation by t-PA occurs in plasma.

Plasmin formed on the fibrin surface has both its lysine-binding sites and active site occupied and is thus only slowly inactivated by \(\alpha\)-antiplasmin (half-life of about 10-100 s compared to 100 ms for free plasmin); while free plasmin, if formed, is rapidly inhibited by \(\alpha\)-antiplasmin.\(^1\) The fibrinolytic process thus seems to be triggered by and confined to fibrin.

### Release and Inhibition of Tissue-Type Plasminogen Activator

Concerning the release of t-PA, the data available at present are not very conclusive. Cash\(^2\)\(^3\) had speculated that t-PA release may be under neurohumoral control and that a plasminogen activator releasing hormone (PARH) would constitute the major pathway for the release from the endothelial cells. This hormone, however, could not be identified in bovine pituitary or hypothalamic extracts.\(^2\)\(^4\) From infusion experiments of bovine protein C in dogs, Comp and Esmon\(^2\)\(^5\) have concluded that activated protein C might be involved in the release of t-PA in vivo. This could, however, not be confirmed using human protein C in squirrel monkeys.\(^2\)\(^6\)

The mechanisms involved in the removal of t-PA from the blood are many and are poorly understood. One main mechanism is through clearance by the liver, which results in a 1/2 of t-PA of a few minutes.\(^2\)\(^7\) With the use of an immunoradiometric assay it appeared that in normal subjects t-PA occurs in the blood in several molecular forms, namely free active
t-PA, and forms with a higher molecular weight identified as t-PA-α2-antiplasmin or t-PA-α1-antitrypsin complexes. 

In several pathological conditions (liver disease, pancreatitis, but not consistently in myocardial infarction or venous thrombosis), up to 50% of the patients develop a (transient) fast, inhibitory activity for t-PA. Gel filtration of plasma samples with added t-PA revealed that this inhibition is associated with the formation of a complex with a M, 110,000-130,000, which could not be identified as a complex of t-PA with any of the known plasma protease inhibitors. From the molecular weight of t-PA (70,000) and of this complex (120,000), a molecular weight estimate of about 50,000 for the inhibitor can be calculated. Assuming formation of a 1:1 stoichiometric complex of t-PA with this inhibitor, researchers have estimated the second-order rate constant at 10^7 M^-1s^-1. A positive correlation was found between inhibition of t-PA and of urokinase. Independent of these studies, several laboratories have recently obtained evidence for the existence of a rapidly acting inhibitor(s) of t-PA at low concentrations in plasma of healthy individuals or at higher levels in pathological plasma samples. In addition, inhibitors of t-PA have been identified in endothelial cell culture fluids and in human platelets. Thus at present, there can be little doubt about the existence of a specific, rapid inhibitor for t-PA in plasma, but its exact pathophysiological role remains to be established.

New Approaches to Thrombolytic Therapy

Efficient thrombolysis in vivo seems to be regulated via adsorption of t-PA and plasminogen on the fibrin surface and in loco generation of plasmin, out of reach of the fast acting α2-antiplasmin in the blood.

The most promising approach to obtain specific thrombolysis, therefore, seems to be the use of fibrin-specific agents. Indeed, streptokinase and urokinase, which have no specific affinity for fibrin, activate circulating and fibrin-bound plasminogen relatively indiscriminately. Plasmin formed in the circulation is immediately neutralized by α2-antiplasmin, and once the inhibitor is exhausted, several plasma proteins are degraded by plasmin (fibrinogen, factor V, factor VIII, etc), causing a bleeding tendency. Therefore, fibrin-specific plasminogen activators such as t-PA, and possibly pro-urokinase, might constitute better thrombolytic agents. The presently available studies on these two agents will be briefly reviewed.

Studies on the Thrombolytic Properties of t-PA

In Vitro Studies

The relative fibrinogenolytic, fibrinolytic, and thrombolytic properties of human t-PA and human urokinase were compared in whole human plasma and in a system composed of a radioactive human blood clot (125I-fibrinogen) suspended in circulating human plasma. It appeared that t-PA has a much higher specific fibrinolytic activity than urokinase. It was possible to dissolve blood clots immersed in circulating human plasma specifically without causing fibrinogenolysis and with only a moderate decrease of plasminogen and α2-antiplasmin; this was not possible with urokinase. Matsson et al., using porcine t-PA in an in vitro system, basically reached the same conclusions.

The specific fibrinolytic effect of human t-PA on human whole blood clots or plasma clots with different degrees of fibrin cross-linking was evaluated in an in vitro system, composed of a 125I-fibrin-labeled clot, suspended in circulating human plasma. After infusion of plasminogen activator (30 IU or 300 ng/ml over 3 hours), non-cross-linked clots lysed more extensively (75% to 100% in 5 hours) than totally cross-linked clots (50% to 65%), while no difference was found between one-chain and two-chain t-PA. The extent of lysis of totally cross-linked human or animal plasma clots suspended in autologous plasma induced by t-PA varied markedly from one species to another, the human system being the most susceptible. Systemic activation of the fibrinolytic system in the circulating plasma was minor and dose-dependent in all species. Similar results were recently obtained using recombinant t-PA.

In some in vitro systems it was shown that lower concentrations of endogenous t-PA (incorporated in a clot) are required to obtain efficient clot lysis as compared to exogenous t-PA (added after clot formation). It was found that the fast reacting t-PA inhibitor was not concentrated into a clot; also t-PA incorporated in a clot is efficiently neutralized by the t-PA inhibitor in the surrounding plasma. Thus, not only do the concentrations of t-PA and of t-PA-inhibitor play a role in the regulation of thrombolysis, but their distribution between the clot and the surrounding plasma are also important.

In Vivo Animal Studies

The thrombolytic effect of t-PA and urokinase was compared in rabbits with an experimental pulmonary embolus. The t-PA caused thrombolysis at lower doses than urokinase (on a molar basis); thrombolysis with t-PA was achieved without extensive plasminogen activation in the circulating blood and without hemostatic breakdown.

In dogs with an experimental thrombosis of the femoral vein, urokinase infusion at a rate of 2,500 IU/kg/hour for 4 hours did not induce significant lysis. With 25,000 IU of urokinase per kg per hour for 4 hours, about 30% lysis was obtained, but this was associated with defibrinogenation. Infusion of 2,500 urokinase equivalent units of t-PA per kg per hour for 4 hours caused 20% to 45% lysis without causing any fibrinogen breakdown.
In a preliminary report, Sampol et al.48 reported successful recanalization with porcine t-PA in dogs with femoral vein thrombosis. Carlin et al.49 induced lysis of intravascular fibrin deposits in the lungs of rats following infusion of human t-PA.

In rabbits with experimental jugular vein thrombosis, the extent of thrombolysis by t-PA is mainly determined by the dose of t-PA and its delivery in the vicinity of the thrombus, and much less by the age of the thrombus or the molecular form of the activator.50

In dogs with a 1- to 2-hour-old anterior descending coronary artery thrombus induced with a copper coil, thrombolysis was induced by intravenous infusion of human t-PA obtained from melanoma cell cultures51 or by recombinant DNA technology.52 In addition to inducing clot lysis, infusion of t-PA also restored intermediary metabolism and nutritional blood flow, without causing systemic fibrinolytic activation.

Gold et al.53 found a linear correlation between the rate of infusion of recombinant t-PA and the time to reperfusion in dogs with a 2-hour-old coronary thrombus. Timely reperfusion was associated with substantial salvage of myocardial tissue and this was done without systemic fibrinogen breakdown.

Flameng et al.54 produced a coronary thrombus in baboons, obtained reperfusion by intravenous administration of recombinant t-PA, and found a linear correlation between the coronary occlusion time and the infarct size.

Buchanan et al.55 used a quantitative bleeding model in rabbits to demonstrate that t-PA, in contrast to streptokinase, did not provoke hemorrhage at thrombolytic doses.

In Vivo Human Studies

The first patients were treated with t-PA in 1981. Intravenous administration of human t-PA (7.5 mg over 24 hours) induced complete lysis of a 6-week-old renal and iliofemoral thrombosis in a renal allograft recipient.56 Thrombolysis was achieved without systemic fibrinolytic activation or hemostatic breakdown, and was not associated with bleeding.

The second case was a 73-year-old man with the nephrotic syndrome, who developed an ascending thrombosis of the iliofemoral vein after removal of an infected femoral-popliteal graft and mid-thigh amputation of the right leg. Venography showed thrombotic masses in the vena cava, and selective venography revealed a thrombus of the right renal vein. The t-PA, 5 mg, given intravenously over 24 hours, resulted in resolution of the thrombosis in the iliac vein, vena cava, and renal vein. No side effects were noted, and again this thrombolytic therapy was not associated with consumption of fibrinogen, plasminogen, α2-antiplasmin or factor V.56

In four patients with deep vein thrombosis over extended segments of the iliac and femoral veins, intravenous infusion of 5 to 15 mg of t-PA over 24 to 36 hours did not, however, result in thrombolysis (unpublished data).

Coronary thrombolysis was induced within 19 to 50 minutes with intravenous or intracoronary infusion of t-PA in six of seven patients with evolving myocardial infarction; this was confirmed angiographically in each case. Circulating fibrinogen, plasminogen and α2-antiplasmin were not depleted. In the one patient in whom lysis was not inducible with t-PA, it was not inducible with streptokinase either.57

From all these studies it thus appears that specific thrombolysis without systemic activation of the fibrinolytic system can be achieved with t-PA. Limited experience in treatment of patients with myocardial infarction suggests that the potentially widely available recombinant t-PA offers a promising practical approach for coronary thrombolysis.

Studies on the Thrombolytic Effect of Pro-Urokinase

Urokinase is a trypsin-like serine protease composed of two polypeptide chains (M, 20,000 and 34,000) connected by a single disulfide bridge. Evidence that urokinase is secreted in an inactive form (pro-urokinase) that can be activated by plasmin, was provided as early as 1973,58 but the mechanism of activation remained unknown. Recently, several groups59-62 have isolated and partially characterized a single-chain form of urokinase.

In vitro, pro-urokinase (pro-UK) is a true enzyme, inactive in plasma but slowly activated in the presence of a fibrin clot.63 The single-chain proenzyme has a higher specific thrombolytic activity and/or a better fibrin selectivity than two-chain urokinase.52,63

In a standardized radioiodinated clot lysis assay, pro-UK purified from a transformed human kidney cell line, was found to lyse clots in a similar way as t-PA, with equivalent efficacy and fibrin specificity.65 Using pro-UK obtained by recombinant DNA technology66 in a system composed of a radioactive human plasma clot immersed in human plasma, Zamarron et al.67 observed a similar fibrinolytic effect of pro-UK and two-chain urokinase, while t-PA caused equivalent degrees of clot lysis of 10-fold lower concentrations. With pro-UK, significant clot lysis could be obtained without systemic activation of the fibrinolytic system. With two-chain urokinase, all concentrations that caused significant clot lysis also caused extensive fibrinolytic activation in the plasma. Whereas t-PA is progressively inactivated upon prolonged incubation in plasma, pro-UK retains its potential fibrinolytic activity for at least 24 hours.63,64 Two-chain urokinase is inactivated in plasma within a few hours.

In rabbits and squirrel monkeys, pro-UK was found57,58 to have an equally short half-life as active urokinase (3 to 6 minutes), due to clearance and degradation by the liver. Its proenzyme nature as such therefore does not result in a prolonged thrombolytic effect in vivo.

Clot lysis induced by pro-UK, as well as by two-chain urokinase and t-PA, is very variable from one
species to another when assayed in an in vitro system consisting of 125I-labeled autologous plasma clots immersed in plasma.69 In general, good reactivity towards t-PA is paralleled by good reactivity towards pro-UK and active urokinase.

Several groups69-71 have compared the thrombolytic effect of single-chain pro-UK and two-chain active urokinase in animal models. By intravenous administration of 3,000 IU of urokinase per kg body weight in dogs with an experimental thrombosis, Sumi et al.70 obtained complete thrombolysis within 1.5 hours with pro-UK, whereas the lysis time was more than 3 hours in the group treated with two-chain urokinase. Gurewich et al.71 studied the thrombolytic effect of pro-UK of human kidney cell origin in rabbits and dogs with pulmonary embolus. In rabbits, the mean extent of thrombolysis after 5 hours was 6%, 17%, and 53% following infusion of saline, two-chain urokinase, and pro-UK, respectively. Infusion of two-chain urokinase was accompanied by systemic fibrinogenolysis, whereas pro-UK did not cause significant fibrinogen degradation. Dogs were found to be about ten times more sensitive to human urokinase than rabbits, but otherwise similar results were obtained as in rabbits.

The thrombolytic properties of recombinant pro-UK (rec-pro-UK), recombinant active urokinase (rec-UK), and natural urinary urokinase (nat-UK) were compared72 in rabbits with a radiolabeled thrombus in the jugular vein. The thrombolytic agents were infused intravascularly over a period of 4 hours and the extent of thrombolysis was measured 2 hours later as the difference between the radioactivity introduced in the clot and that recovered in the vein segment at the end of the experiment. Significant thrombolysis with nat-UK and rec-UK was only obtained with 240,000 IU/kg or more, and this was associated with a marked systemic activation of the fibrinolytic system, as evidenced by consumption of plasminogen and α2-antiplasmin and fibrinogen breakdown. Infusion of rec-pro-UK induced thrombolysis at a dose of 60,000 IU/kg or more and without associated activation of the fibrinolytic system. However, t-PA induced a comparable degree of thrombolysis without systemic activation of the fibrinolytic system at two- to fourfold lower concentrations. Although this difference could result from different reactivity of the rabbit toward both types of fibrinolytic agents, our findings obtained in a human plasma system in vitro73 seem to support the conclusion that t-PA probably also has a higher specific thrombolytic activity in humans.

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